Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/AU05/000041

International filing date: 14 January 2005 (14.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: AU
Number: 2004903914
Filing date: 16 July 2004 (16.07.2004)

Date of receipt at the International Bureau: 08 February 2005 (08.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

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Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

PCT/AU2005/000041

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First day of February 2005

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AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: "CHAPERONIN 10 MODULATION OF CYTOKINE AND CHEMOKINE SECRETION "

The invention is described in the following statement:

TITLE

CHAPERONIN 10 MODULATION OF CYTOKINE AND CHEMOKINE SECRETION

FIELD OF INVENTION

This invention relates to a method of modulating immunomodulator secretion in immune cells and treatment of diseases and disorders resulting from excessive immunomodulator secretion. More particularly, this invention relates to a method of modulation of cytokine and chemokine secretion using chaperonin 10 through interaction with Hsp60 protein, Toll-like receptors and/or β1-integrins that mediate cytokine and chemokine secretion.

BACKGROUND OF THE INVENTION

Mammalian chaperonin 10 (also known as heat shock protein 10) and heat shock protein 60 (Hsp60) are mitochondrial proteins involved in protein folding, and are homologues of the bacterial proteins GroES and GroEL, respectively. GroES and chaperonin 10 (Cpn10) oligomerise into seven member rings that bind as a lid onto a cup-like structure comprising seven GroEL or Hsp60 molecules, which tether the denatured proteins (Fiaux *et al.*, 2002, Nature, 418, 207-211; Meyer *et al.*, 2003, Cell, 13, 369-381). Hsp10 and Hsp60 are also frequently found at the cell surface (Belles *et al.*, 1999, Infect Immun, 67, 4191-4200; Feng *et al.*, 2001, Blood, 97, 3505-3512) and in the extracellular fluid (Michael *et al.*, 2003, J Biol Chem, 278, 7607-7616; Johnson *et al.*, 2003, Cir Rev Immunol, 23, 15-44).

HSPs released from dying or otherwise stressed cells are believed to be a source of "danger" signals informing the innate and adaptive immune system of the presence of tissue damage induced by various insults including infection, injury, toxins, heat and/or cellular stress (Johnson *et al.*, 2003, Crit Rev Immunol, 23, 25-44; Wallin *et al.*, 2002, Trends Immunol, 23, 130-135; Beg, 2002, Trends Immunol, 23, 509-512). Human studies have also shown an association between elevated serum Hsp60 and low socioeconomic status, social isolation, psychological distress and increased levels of the pro-inflammatory cytokine, TNF-α (Lewthwaite *et al.*, 2002, Circulation, 106, 196-201).

The Toll-like receptor family plays an important role in inflammation and immunity in insects, animals and plants. Toll-like receptors (TLRs) are expressed on cells of the mononuclear lineage including lymphocytes, macrophages and dendritic cells. TLR activation by pathogens induces intracellular signaling that primarily results in activation of the transcription factor NF-κB (Beg, *supra*), and modulation of cytokine production. However, a series of other pathways can also be triggered, including p38 mitogen activated kinase, c-Jun-N-terminal kinase and extracellular signal related kinase pathways (Flohe *et al., supra*; Triantafilou & Triantafilou, *supra*). The patterns of gene expression induced by ligation of the different TLRs are distinct but often overlap. For instance a large proportion of the genes upregulated by TLR3 agonists and double stranded RNA are also upregulated by TLR4 agonists and LPS (Doyle *et al.*, 2002, Immunity, 17, 251-263). TLR4 activation by LPS in macrophages results in TNF-α, IL-12 IL-1β, RANTES and MIP1β secretion (Flohe *et al., supra*; Jones *et al.*, 2002, J Leukoc Biol, 69, 1036-1044).

Host Hsp60, Hsp70 and chaperone gp96 molecules are believed to activate the innate and adaptive immune system via TLR2 and TLR4 (Flohe, *et al.*, 2003, J Immunol, 170, 2340-2348; Beg, *supra*; Zanin-Zhorov *et al.*, 2003, Faseb, J17, 1567-1569). TLR2 is also activated by TLT2 agonists, lipoteichoic acid and lipopeptides, which can be components of the outer wall of certain bacteria. TLR4 is also activated by lipoproteins or lipopolysaccharide (LPS) or endotoxin, which is a component of the outer wall of gram-negative bacteria. It is unknown how HSPs interact with and activate TLRs however, it has been reported that HSPs, including Hsp60, can bind to the surface of cells (Beg, *supra;* Habich *et al.*, 2003, FEBS Lett, 533, 105-109). HSPs may form part of a multi-component TLR complex and somehow influence LPS signaling (Triantafilou & Triantafilou, 2002, Trends Immunol, 23, 301-304). It is also possible that HSPs (particularly Hsp60) may play a role in potentiating TLR signaling by low or sub-threshold levels of LPS (Johnson *et al.*, *supra*).

Cpn10 was originally identified as a suppressive factor present early in pregnancy and has shown immunosuppressive activity in experimental autoimmune encephalomyelitis, delayed type hypersensitivity and allograft rejection models (Zhang *et al.*, 2003, J Neurol Sci, 212, 37-46; Morton *et al.*, 2000, Immunol Cell Biol, 78, 603-607).

A recent study using *Mycobacterium tuberculosis* Cpn10 described in International Publication WO 02/40038, suggests that this molecule may have efficacy in treating disorders such as cancer, allergic reactions and/or conditions mediated by Th2-type immune responses. It is proposed that this may be achieved through induction of cytokines such as TNFα and IL-6.

However, the mechanism of action by which eukaroytic Cpn10, and in particular mammalian Cpn10, exerts its immunosuppressive effects has remained obscure.

SUMMARY OF INVENTION

Surprisingly, the inventors have demonstrated that mammalian Cpn10 modulates Toll-like receptor agonist-mediated stimulation of immunomodulator secretion through Toll-like receptors and/or β1-integrins demonstrated by a reduction of TNF-α, interleukin 6 (IL-6) and RANTES secretion and NF-κB activation. Cpn10 also independently promotes IL-10 production.

The invention in one broad form is directed to the modulation of Toll-like receptor and/or β1-integrin signaling by chaperonin 10 (Cpn10) to thereby modulate immunomodulator secretion. Although not wishing to be bound by any particular theory, the inventors also propose that Cpn10 inhibition of Toll-like receptor and/or β1-integrin signaling may be mediated by Hsp60.

In another broad form the invention is directed to use of Cpn10 for modulating immunomodulator secretion.

In a first aspect, the invention provides a method of regulating immunomodulator secretion in an animal, or in one or more cells, or tissues or organs derived therefrom, including the step of administering Cpn10, or a derivative of Cpn10, to the animal, cells, tissues or organs, to thereby modulate Toll-like receptor signaling and thereby regulate immunomodulator secretion.

In a second aspect, there is provided a method of regulating immunomodulator secretion in an animal, or in one or more cells, or tissues or organs derived therefrom including the step of administering Cpn10, or derivative

of Cpn10, to the animal, cells, tissues or organs, to thereby modulate Hsp60 protein activity and thereby regulate immunomodulator secretion.

In a third aspect, the invention provides a method of regulating immunomodulator secretion in an animal, or in one or more cells, or tissues or organs derived therefrom, including the step of administering Cpn10, or a derivative of Cpn10, to the animal, cells, tissues or organs, to thereby modulate β1-integrin signaling and thereby regulate immunomodulator secretion.

According to these aspects, the invention provides a method of modulating immunomodulator secretion to thereby modulate the immune response in an animal in response to acute or chronic inflammatory diseases such as septic shock, inflammatory bowel disease, arthritis, psoriasis, heart disease, atherosclerosis, chronic pulmonary disease, cachexia, multiple sclerosis, GVHD, transplantation and cancer.

Suitably, secretion of the immunomodulator is inducible by a Toll-like receptor agonist.

Preferably, the Toll-like receptor is selected from the group consisting of TLR2, TLR3 and TLR4.

Preferably, the Toll-like receptor agonist is selected from the group consisting of LPS, lipopeptide, double stranded RNA and Hsp60.

Preferably, the lipopeptide is PAM_3CysSK_4.

In one embodiment, where the Toll-like receptor is TLR4, the agonist is LPS and/or Hsp60.

In another embodiment, where the Toll-like receptor is TLR3, the agonist is double stranded RNA.

In yet another embodiment, where the Toll-like receptor is TLR2, the agonist is PAM_3CysSK_4 and/or Hsp60.

Preferably, the cell is an immune cell.

More preferably, the immune cell is a T cell, monocyte, macrophage or a lymphocyte.

Preferably, the animal is a mammal.

More preferably, the mammal is a human.

According to the aforementioned aspects, in one embodiment, the immunomodulator is a pro-inflammatory cytokine, such as TNF-α or IL-6, or a pro-inflammatory chemokine, such as RANTES.

In another embodiment, the immunomodulator is an anti-inflammatory cytokine, such as interleukin-10, or anti-inflammatory chemokine, such as TGF-β.

In embodiments where the immunomodulator is a pro-inflammatory cytokine or chemokine, administration of Cpn10 preferably inhibits, suppresses or otherwise reduces secretion of said immunomodulator.

In embodiments where the immunomodulator is an anti-inflammatory cytokine or chemokine, administration of Cpn10 preferably augments, facilitates or otherwise increases secretion of said immunomodulator.

In a fourth aspect, the invention provides use of Cpn10 to produce or design a modulator which modulates Toll-like receptor signaling and/or Toll-like receptor agonist-inducible immunomodulator secretion.

Preferably, the modulator modulates a Toll-like receptor.

Preferably, the Toll-like receptor is selected from the group consisting of TLR2, TLR3 and TLR4.

More preferably, the Toll-like receptor is TLR4.

In a fifth aspect, the invention contemplates use of Cpn10 to produce or design a modulator of Hsp60 which modulates Hsp60-dependent Toll-like receptor signaling and/or Toll-like receptor agonist-inducible immunomodulator secretion.

Preferably, the modulator modulates the Hsp60-dependent augmentation of Toll-like receptor agonist signaling and immunomodulator secretion.

In one embodiment of this aspect, said Hsp60 modulator can bind to a Hsp60 molecule preventing Hsp60 binding to, interacting with or stimulating a Toll-like receptor or other cell surface receptor(s).

Preferably, said Hsp60 modulator prevents Hsp60 from mediating or contributing to Toll-like receptor signaling.

In another embodiment of this aspect, said Hsp60 modulator can bind to a Toll-like receptor, thereby preventing Hsp60 binding to, interacting with or stimulating said Toll-like receptor.

Preferably, said Hsp60 modulator prevents Hsp60 from mediating or contributing to Toll-like receptor signaling.

In a sixth aspect, the invention provides use of Cpn10 to produce or design a modulator of β1-integrin signaling.

In a seventh aspect, the invention contemplates use of Cpn10 to produce or design a modulator of Hsp60 which modulates Hsp60-dependent β1-integrin signaling and/or β1-integrin agonist-inducible immunomodulator secretion.

Preferably, the modulator modulates the Hsp60-dependent augmentation of β1-integrin agonist signaling and immunomodulator secretion.

In one embodiment of this aspect, said Hsp60 modulator can bind to a Hsp60 molecule preventing Hsp60 binding to, interacting with or stimulating a β1-integrin.

Preferably, said Hsp60 modulator prevents Hsp60 from mediating or contributing to β1-integrin signaling.

In another embodiment of this aspect, said Hsp60 modulator can bind to a β1-integrin receptor, thereby preventing Hsp60 binding to, interacting with or stimulating said β1-integrin.

Preferably, said Hsp60 modulator prevents Hsp60 from mediating or contributing to β1-integrin signaling.

It will be appreciated, according to the aforementioned aspects, that the modulator may be an agonist, antagonist or blocker.

In an eighth aspect, the invention provides an isolated protein complex comprising Cpn10 and Hsp60.

In one form the isolated protein complex is obtainable from a cell surface or extracellular fluid.

In a ninth aspect, the invention provides an isolated protein complex comprising Cpn10, Hsp60 and a Toll-like receptor.

In a tenth aspect, the invention provides an isolated protein complex comprising Cpn10, Hsp60 and a β1-integrin.

In an eleventh aspect, the invention provides an isolated protein complex comprising Cpn10, Hsp60, a β1-integrin and a Toll-like receptor.

In one embodiment of the eighth to eleventh aspects, the invention

provides use of the isolated protein complex to produce or design an agent capable of regulating immunomodulator secretion.

In one particular embodiment said agent suppresses, reduces or otherwise inhibits pro-inflammatory cytokine or chemokine secretion normally induced by a Toll-like receptor agonist.

In another embodiment said agent augments, facilitates or otherwise increases anti-inflammatory cytokine or chemokine secretion normally induced by a Toll-like receptor agonist.

In a twelfth aspect, the invention provides a pharmaceutical composition, when used according to any one of the first to third aspects, comprising Cpn10 and a pharmaceutically acceptable carrier, diluent or excipient.

In a particularly preferred form, the pharmaceutical composition is a composition capable of controlled release of Cpn10.

In a thirteenth aspect, the invention provides a method of inhibiting, suppressing or otherwise reducing secretion of IL-6 and/or RANTES in an animal, or in one or more cells, tissues or organs derived therefrom, including the step of administering Cpn10, or a derivative of Cpn10, to the animal, cells, tissues or organs, to thereby inhibit suppress or otherwise reduce secretion of IL-6 and/or RANTES by said animal or by the one or more cells, tissues or organs derived therefrom.

Throughout this specification, "comprise", "comprises" and "comprising" are used inclusively rather than exclusively, will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Cpn10 inhibits LPS-induced activation of RAW264.7 cells and pro-inflammatory mediator production.

(A) Cpn10-mediated inhibition of LPS-induced NF-κB activity. In 9 separate experiments 100 μg/ml of Cpn10 (Cpn10 +) or buffer (Cpn10 -) was preincubated with RAW264-HIV-LTR-LUC cells for 2 h. LPS was then added at 5, 1 and 0.2 ng/ml and luciferase activity measured 2 h later. The relative light units (RLU) of luciferase obtained for 5 ng/ml of LPS was set at 100% relative luciferase activity, and 0% represents the RLU obtained in the absence of LPS. Cpn10 alone did not stimulate significant RLU (data not shown). The mean percentage reduction (± SD) in RLU mediated by Cpn10 is indicated for each concentration of LPS, and the significance calculated using a paired t test.

(B) Cpn10-mediated inhibition of LPS-induced TNF-α secretion. RAW264.7 cells were incubated with 0.5 ng/ml of LPS in the presence of 20 μg/ml of Cpn10 (Cpn10 +) or buffer (Cpn10 -) and after 4 hours the supernatant was analysed for TNF-α by ELISA; (eight separate experiments are shown). The mean percentage reduction (± SD) in TNF-α secretion and the significance, calculated using a paired t test, is indicated.

(C) Cpn10-mediated inhibition of LPS-induced RANTES secretion. RAW264.7 cells were incubated with 5 ng/ml of LPS in the presence of 100 μg/ml of Cpn10 and after 4 hours the supernatant was analysed for RANTES by ELISA; (seven separate experiments are shown). The percentage reduction and significance were calculated as for B.

FIG. 2 Effect of Cpn10 on cytokine secretion in murine systems.

(A) Cpn10 treatment reduced capacity of LPS-stimulated peritoneal macrophages to produce TNF-α. C57BL/6 mice (n=3) were treated with Cpn10 (Cpn10 +) or control diluent (Cpn10 -). Peritoneal macrophages were harvested by peritoneal lavage on day 6 and pooled from individual animals within the treatment group. Cells were plated at $2x10^5$/well in the presence of LPS (1 μg/ml). Culture supernatants were collected at 5 hours and levels of TNF-α were assessed by ELISA. (Wells without LPS produced no detectable TNF-α - data not shown). Mean ± SE of triplicate wells are shown, and are normalized to production per 10^5 macrophages based on CD11b staining. Data from two identical experiments are shown and the average percentage reduction is indicated with the significance calculated by ANOVA.

(B) Cpn10 treatment augmented IL-10 production from splenocytes. C57BL/6 mice were treated with either Cpn10 or control diluent as above. Splenocytes were harvested on day 6 and pooled from individual animals within a treatment group and cultured at $5x10^5$/well in the presence of LPS (10 μg/ml). Culture supernatants were collected at 48 hours and levels of IL-10 determined by ELISA. Means ± SE of triplicate wells are shown. Average percentage increase is shown with statistics calculated as for A.

(C) Cpn10 treatment reduced TNF-α production from $IL10^{-/-}$ peritoneal macrophages. $IL10^{-/-}$ C57BL/6 mice were treated with Cpn10 or control diluent as above and peritoneal macrophages were harvested as for A. After 5 hours of culture in the presence of LPS (0.1, 1 or 10 μg/ml) TNF-α

was determined in culture supernatants by ELISA. Mean ± SE of triplicate wells for one representative experiment is shown. TNF-α levels were compared for Cpn10 treated and control animals using a non-parametric t test.

FIG. 3 Cpn10-treatment of human peripheral blood mononuclear cells (PBMC) reduces LPS-induced TNF-α secretion.

(A) The LPS-induced TNF-α levels secreted by PBMC cultures were determined in 10 separate experiments using PBMC from 7 different donors. Cpn10 (400 μg/ml) was added to the PBMC 2 hours prior to the addition of LPS. The percentage reduction and significance were calculated as for Fig. 2B.

(B, C) Dose-dependent Cpn10-mediated reduction in TNF-α and increase in IL-10 secretion after LPS stimulation. A representative experiment using PBMC from a single donor is shown using a concentration range of Cpn10 and LPS. Supernatants were removed after 4 hrs and analysed for TNF-α (B) and IL-10 (C).

FIG. 4 Cpn10 activity in murine inflammatory models.

(A) Cpn10 reduces LPS-induced serum TNF-α and RANTES levels and increases IL-10 levels. In 5 separate experiments C57BL/6 mice (n=3 or 4 per group) were given buffer (Cpn10 -) or 100 μg of Cpn10 (Cpn10 +) iv 30 mins before iv administration of 10 μg of LPS. After 1.5 hours the animals were sacrificed and serum TNF-α, RANTES and IL-10 levels determined; (the latter two were assessed in 3/5 experiments). Error bars represent standard errors within each experiment. The percentage

reduction in TNF-α and RANTES and increase in IL-10 (± SD) is indicated and the significance calculated using ANOVA tests.

(B) Pre-transplant treatment with Cpn10 delays GVHD mortality and reduces clinical severity of acute disease. Syngeneic negative controls (n=8) (white circles) represent B6D2F1 mice transplanted with syngeneic B6D2F1 bone marrow and T cells. Allogeneic positive controls (n=10) (white squares) represent diluent pre-treated B6D2F1 recipient mice transplanted with cells from diluent pre-treated B6 donor mice. Allogeneic + Cpn10 (n=10) (black squares) represent B6D2F1 recipients receiving bone marrow and T cell grafts from B6 donor mice where both recipients and donors were pre-treated with Cpn10 prior to transplantation. Kaplan-Meier survival curves and clinical scores are shown for the three groups and the allogeneic groups treated with and without Cpn10 compared by Log Rank Statistic and non-parametric t test, respectively. Clinical scores were only significantly different on day 7.

FIG. 5 Cpn10 activity is blocked by anti-Hsp60 antiserum. RAW264-HIV-LTR-LUC cells were treated with anti-Hsp60 or control serum for 1 h, and then Cpn10 (100 μg/ml) (Cpn10+) or buffer (Cpn10-) was added for a further 2 h. LPS was then added and luciferase activity measured 2 h later. The mean percentage reduction (± SD) in relative light units is indicated for 5, 1 and 0.2 ng/ml LPS taken together, and the significance calculated using a 2 way ANOVA, which included a term for LPS concentration.

FIG. 6 Western analysis of the cell surface profile of Cpn10 and control treated A549 cells. (A and B). 12.5% PAGE of streptavidin-purified biotinylated

cell surface proteins. Lane 1 and 2, Cpn10 treated A549 cells. Lane 3 and 4, untreated control A549 cells. Lanes 1 and 3 contain 1 fifth of the protein loaded into lanes 2 and 4. (A) The membrane shown in (B) was stripped and re-probed with an anti-actin monoclonal antibody and was over exposed for 30 mins. A very faint band at 42 Kd (the mol. wt. of actin) could be detected (arrow). (B) Probing with rabbit anti-Cpn10 antibody (2 min exposure). (C) Same membrane as A and B stripped and re-probed with normal rabbit serum at the same dilution as B (1/500). (D) 10% PAGE of streptavidin purified biotinylated cell surface proteins. Lanes 1, 3 and 5 untreated control A549 cells. Lanes 2, 4 and 6 Cpn10 treated A549 cells; (Loading as for 2 and 4 above). Lanes 3/4 and 5/6 are from the same Western, the latter exposed for 30 sec the former 2 mins. Lanes 1 and 2 probed with goat anti-Hsp60, lanes 3-6 rabbit anti-Hsp60.

FIG.7 (A) Area of subcutaneous granuloma present in BALB/c mice given CFA with and without Cpn10 treatment. (B-E) PAM3CysSK$_4$-induced macrophage activation is inhibited by Cpn10 in Raw 264 -HIV LTR Luc cells. (B) Cpn10 or diluent added for 2 hrs followed by addition of PAM3CysSK$_4$ for 2 h followed by the luciferase assay. (C) Cpn10 or diluent added for 2 hrs, followed by washing to remove Cpn10 or diluent, followed by addition of PAM3CysSK$_4$ for 2 h and then the luciferase assay. (D) Cpn10 or diluent added for 2 hrs followed by addition of PAM3CysSK$_4$ for 2 h and then the luciferase assay. (E) The same protocol was used in (B) except the cells were activated with LPS instead of

PAM3CysSK$_4$. The top panel shows the relative light unit data, and the bottom the percent inhibition mediated by Cpn10.

FIG. 8 Inhibition of polyIC/TLR3 mediated HIV LTR activation compared with inhibition of LPS/TLR4 inhibition by Cpn10. Note the % inhibition of RLU without Cpn10 were set to zero.

FIG. 9 Mean (± SEM) weight loss during adjuvant arthritis (n=10 per group).

FIG. 10. Cpn10-treatment of human peripheral blood mononuclear cells reduced LPS-induced TNF-α and IL-6 secretion. (A) Hsp10 reduced LPS-induced TNF-α secretion. Hsp10 (1 and 10 μg/ml) or buffer (-) was added to PBMC from five different donors 1 h prior to the addition of 0.04 ng/ml of LPS. Supernates were removed after 6 h and analyzed for TNF-α. The percentage reductions and significance are indicated; the latter were calculated using a paired t test. For all donors 10 ug/ml of Hsp10, in the absence of LPS, failed to induce TNF-α levels above the level of detection (31 pg/ml). (B) Pre-treatment with LPS for 1 h was unable to induced tolerance to subsequent LPS-induced TNF-α secretion. PBMC from three donors (1, 2 and 3) were exposed to the indicated LPS pre-treatment concentrations. After 1 h the PBMC were stimulated with 0.04 ng/ml of LPS, and the supernates analyzed for cytokine 6 h later. (C) Hsp10 reduced LPS-induced IL-6 secretion. Hsp10 (1 and 10 μg/ml) or buffer (-) was added to PBMC from eight different donors 1 h prior to the addition of 0.04 ng/ml of LPS. Supernates were removed after 20 h and analyzed for IL-6. The percentage reductions and significance (calculated

as for A) are indicated. For all donors 10 ug/ml of Hsp10, in the absence of LPS, failed to induce IL-6 levels above the level of detection (9 pg/ml). (D) Pre-treatment with LPS for 1 h was unable to induced tolerance to subsequent LPS-induced IL-6 secretion. PBMC from a donor were exposed to the indicated LPS pre-treatment concentrations. After 1 h the PBMC were stimulated with 0.04 ng/ml of LPS, and the supernates analyzed for cytokine 20 h later.

FIG. 11. Cpn10 inhibited LPS-induced activation of RAW264.7 cells and pro-inflammatory mediator production. (A) Cpn10-mediated inhibition of LPS-induced NF-kB activity. In nine separate experiments 100 μg/ml of Cpn10 (Cpn10 +) or buffer (Cpn10 -) was pre-incubated with RAW264-HIV-LTR-LUC cells for 2 h. LPS was then added at 5, 1 or 0.2 ng/ml and luciferase activity measured following 2 h incubation. The relative light units (RLU) of luciferase obtained with 5 ng/ml of LPS (in the absence of Cpn10) was set at 100% relative luciferase activity, and 0% represents the RLU obtained in the absence of LPS. Cpn10, in the absence of LPS, did not stimulate significant luciferase activity (data not shown). The mean percentage reduction (± SD) in RLU mediated by Cpn10 is indicated for each concentration of LPS, and the significance calculated using a paired *t* test. (B) Trypsin-treated Cpn10 failed to inhibit LPS-induced NF-kB activity. Treatment of RAW264-HIV-LTR-LUC cells in duplicate for 2 h with 100 ug/ml Cpn10 (Cpn10) significantly reduced the RLU induced by 5, 1 and 0.2 ng/ml LPS compared with cells treated with buffer (Control); percent reductions in RLU after subtraction of background were 29.7 ± 0.8

(SD), 50 ± 4.6, and 71 ± 7.7, respectively ($p<0.001$ by two factor ANOVA, which included a term for LPS concentration). Compared with the Control, treatment with trypsin-treated Cpn10 (Trypsin Cpn10) gave 0.1 ± 8.8, 11.6 ± 4.2, and 21 ± 7.4, and trypsin-treated buffer (Trypsin Buffer) 1.4 ± 2.1, 5.8 ± 1.1, and 14.9 ± 2.4 percent reduction for 5, 1 and 0.2 ng/ml LPS, respectively; (neither were significantly different from Control or each other). Trypsin treatment of the stimulating LPS did not affect LPS activity (Trypsin LPS, $p>0.05$). (C). LPS pre-treatment 2 h prior to LPS stimulation did not inhibit LUC activity. RAW264-HIV-LTR-LUC cells were pre-treated with the indicated LPS concentrations in duplicate, after 2 h the cells were stimulated with 5, 1, 0.2 and 0 ng/ml LPS and the LUC activity measured 2 h later. (D) Cpn10-mediated reduction in LPS-induced LUC activity is dose responsive. The experiment was set up as in Fig. 3*A* except the Cpn10 concentration was varied as indicated. For each LPS concentration the percent inhibition in LUC activity over control cells not pre-treated with Cpn10 is indicated. (E) Cpn10-mediated inhibition of LPS-induced RANTES secretion. RAW264.7 cells were simultaneously treated with 0, 0.125 or 0.25 ng/ml of LPS and the indicated concentration of Cpn10. After 6 h the supernate was analyzed for RANTES by ELISA. The mean percentage reduction in RANTES secretion is indicated.

FIG. 12. Intravenous infusion, but not subcutaneous injection, of Cpn10 induces changes in the magnitude of the LPS-driven TNF-α response by PBMC *in vitro*. A: LPS-driven TNF-α production on day 0 (~12 hr pre-infusion)

and day 1 (8 hr post-infusion). B: Data from Figure 1A graphed as the change in LPS-driven TNF-α production from pre- to post-infusion. C: LPS-stimulated TNF-α production on day 0 (~12 hr pre-injection) vs. day 1 (8 hr post-subcutaneous injection Cpn10/placebo).

FIG.13 Intravenous infusion of Cpn10 induces changes in the magnitude of the LPS-driven TNF-α response by PBMC *in vitro:* LPS-driven TNF-α production from PBMC isolated on day 0 (~12 hr pre-infusion) compared with cells isolated on days 1, 4, and 5 (approx. 8 hr post-infusion). In addition, PBMC isolated on days 8 and 12 (i.e. 3 days and 7 days following the final day 5 Cpn10 infusion, respectively) were compared with the day 0 LPS-stimulated response. Cell culture supernatants were also tested for production of IL-6 and demonstrated the same overall trend as depicted here for TNF-α.

FIG. 14. C57Bl/6 mice were treated for 5 days with Cpn-10 or control buffer administered via osmotic pumps (OP) or subcutaneously (SC) (n=9 per group). Results from 3 identical experiments are presented as percentage change from the corresponding buffer control. Primary MLC (A) and secondary MLC (B): stimulation with anti CD3/CD28 mAb, allogeneic (F1) macrophages and ConA. C) TNFα from LPS stimulated macrophages and D) IL-10 from LPS stimulated splenocytes.

DETAILED DESCRIPTION OF INVENTION

The present inventors discovered that, in a number of different human and murine *in vitro* and *in vivo* systems, Cpn10 inhibits LPS-mediated secretion of the

pro-inflammatory cytokines TNF-α and IL-6 and the pro-inflammatory chemokine RANTES, and increases LPS-induced secretion of the anti-inflammatory cytokine IL-10 in monocytes, macrophages and lymphocytes.

This is in contrast to International Publication WO 02/40038 which proposes that *Mycobacterium* Cpn10 acts to suppress Th2-mediated immune responses, by increasing expression of cytokines such as TNF-α and IL-6.

According to the present invention, Cpn10 acts neither via a Th1- nor a Th2-dependent mechanism.

The inventors have unexpectedly demonstrated that Cpn10 modulates immunomodulator secretion by modulating intracellular signaling by TLR4, TLR3 and TLR2 agonists. The inventors believe Cpn10 binds Hsp60, preventing Hsp60 interacting with, or contributing to TLR4, TLR3 and TLR2 signaling, and thereby inhibiting Hsp60-mediated augmentation of TLR signaling.

Hsp60 is also reported to bind to β1 integrin (Iwata *et al.*, 2000, J Dermatol Phy, 23,75-86). β1 integrin is capable of mediating signal transduction and β1 integrin-activation can alter the behavior of cells in response to inflammatory stimuli (Zanin-Zhorov, *supra*). Thus Hsp60-mediated signaling via β1 integrin may augment TLR-mediated signaling. The inventors believe Cpn10 may also inhibit β1 integrin augmentation of TLR-mediated signaling by binding to Hsp60.

Cpn10 reduces LPS-stimulated NFκB activation, and TNF-α and RANTES secretion, and increases IL-10 secretion in a dose-dependent manner in human and mouse cell lines and freshly isolated cells *in vitro*.

Cpn10 reduces TNF-α and RANTES production and increases IL-10 production in an *in vivo* non-lethal endotoxemia model in mice. Cpn10 also has significant immunosuppressive activity in an *in vivo* mouse transplantation model and Cpn10 treatment increases the survival rate of mice suffering from graft versus host disease.

Cpn10 also reduces cachexia in rats suffering from adjuvant-induced arthritis. Elevated levels of inflammatory cytokines are associated with cachexia in a number of diseases, such as cancer and rheumatoid arthritis.

In addition Cpn10 improves wound healing in an *in vivo* mouse model. The invention also shows that Cpn10 administered as a single intravenous dose to humans *in vivo*, markedly reduces the pro-inflammatory cytokine response following LPS-stimulation *ex vivo* in a dose-responsive manner clearly demonstrating that Cpn10 has immunomodulatory effects in human clinical trial subjects.

Excessive inflammation or uncurtailed immune responses are detrimental to a host, hence a number of negative feedback systems have evolved to dampen production of pro-inflammatory mediators. One such negative feedback mechanism includes IL-10, an important immunoregulatory cytokine secreted by mononuclear cells and monocytes, which is involved in limitation of inflammatory responses and induction of immune tolerance.

Cpn10 inhibits but does not abolish TNF-α, IL-6 and RANTES secretion. This is a desirable characteristic since complete TNF-α removal (for example, by anti-TNF-α antibodies) can result in compromised immunity, predisposing

patients to infection, and reduced tumour surveillance, predisposing patients to increased frequency of tumours.

The ability of Cpn10 to reduce secretion of immunomodulators indicates that Cpn10 will find therapeutic application in conditions where excessive pro-inflammatory immunomodulator secretion leads to disease.

Many diseases are associated with excessive or chronic inflammation, hence modulation of TLR receptor signaling resulting in modulation of cytokine secretion may have widespread clinical benefits. For example, excessive secretion of pro-inflammatory cytokines, such as TNF-α is one of the leading causes of death in acute conditions such as septic shock, and it is one of the main factors contributing to ongoing tissue damage in chronic inflammatory diseases such as inflammatory bowel disease (IBD), arthritis, psoriasis, congestive heart disease, multiple sclerosis, and chronic obstructive pulmonary disease.

In tissue or organ transplantation the host or donor lymphocytes can recognise the donor or host cell antigens, respectively, as foreign and release cytokines which activate cells of the innate immune system resulting in rejection of the transplanted tissue or organ or graft versus host disease. Immunosuppressive drugs play a large role in the therapeutic treatment and management of transplant rejection and graft versus host disease. However, the drugs evoke severe side effects in patients, they are very expensive and in some patients they are poorly effective.

For the purposes of the invention, by "*immunomodulator*" is meant a molecular mediator secreted by cells of the immune system or a molecular mediator which interacts with cells of the immune system that plays a role in

activation, maintenance, maturation, inhibition, suppression or augmentation of an immune response.

By "*cytokine*" is meant a molecular mediator secreted by cells of the immune system that plays a role in activation, maintenance, maturation, inhibition, suppression or augmentation of an immune response. Non-limiting examples of cytokines are TNF-α, IL-6, interleukin-1 and interleukin-10.

By "*chemokine*" is meant a molecular mediator that acts to promote and/or regulate cell migration and activation. Non-limiting examples of a chemokine is RANTES.

By "*pro-inflammatory immunomodulator*" is meant a cytokine or chemokine that plays a role or has some involvement in an inflammatory process or inflammatory response.

By "*anti-inflammatory immunomodulator*" is meant a cytokine or chemokine that plays a role in inhibiting, suppressing or otherwise decreasing an inflammatory response.

By "*immunosuppressive agent*" is meant an agent that can prophylactically or therapeutically suppress an immune response or an autoimmune response, for example, against a transplanted allogeneic or xenogeneic cell, tissue or organ.

By "*isolated*" is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together

with components that normally accompany it in its natural state. Isolated material may be in native, chemical synthetic or recombinant form.

By "*protein*" is meant an amino acid polymer. The amino acids may be natural or non-natural amino acids D- and L- amino acids, as are well understood in the art.

A "*peptide*" is a protein having no more than fifty (50) amino acids.

A "*polypeptide*" is a protein having more than fifty (50) amino acids.

The term "*nucleic acid*" as used herein designates single-or double-stranded mRNA, RNA, cRNA, RNAi and DNA inclusive of cDNA and genomic DNA.

Cpn10and Cpn10 fragments, variants and derivatives

According to the present invention, "*Cpn10*" or "*chaperonin 10*" refers to any eukaryotic Cpn10, including mammalian Cpn 10 such as human, mouse, rat and other forms of Cpn10. Cpn10 protein may comprise naturally occurring modification such as glycosylation or acetylation and/or be in native, chemical synthetic or recombinant form. It will also be appreciated that Cpn10 may be referred to as "*Hsp10*". These are to be treated as referring to the same protein.

In one embodiment, a "*fragment*" includes an amino acid sequence that constitutes less than 100%, but at least 20%, preferably at least 30%, more preferably at least 80% or even more preferably at least 90%, 95% or 98% of a Cpn10 protein.

The term "*fragment*" includes and encompasses a "*biologically active fragment*", which retains a biological activity of a Cpn10 protein. For example, a biologically active fragment of Cpn10 capable of inducing immunosuppression in

a subject may be used in accordance with the invention. The biologically active fragment constitutes at least greater than 1% of the biological activity of the entire Cpn10 protein, preferably at least greater than 10% biological activity, more preferably at least greater than 25% biological activity, even more preferably at least greater than 50% biological activity and advantageously at least 70%, 80%, 90% or at least 95% of a biological activity of Cpn10.

As used herein, *"variant"* proteins are proteins in which one or more amino acids have been replaced by different amino acids. Protein variants of Cpn10 that retain biological activity of native or wild type Cpn10 may be used in accordance with the invention. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the protein (conservative substitutions). Generally, the substitutions which are likely to produce the greatest changes in a polypeptide's properties are those in which (a) a hydrophilic residue (*e.g.*, Ser or Thr) is substituted for, or by, a hydrophobic residue (*e.g.* Leu, Ile, Phe or Val); (b) a cysteine or proline is substituted for, or by, any other residue; (c) a residue having an electropositive side chain (*e.g.*, Arg, His or Lys) is substituted for, or by, an electronegative residue (*e.g.*, Glu or Asp) or (d) a residue having a bulky side chain (*e.g.*, Phe or Trp) is substituted for, or by, one having a smaller side chain (*e.g.*, Ala, Ser) or no side chain (*e.g.*, Gly).

With regard to protein variants, these can be created by mutagenising a Cpn 10 protein or by mutagenising an encoding nucleic acid, such as by random mutagenesis or site-directed mutagenesis. Examples of nucleic acid mutagenesis methods are provided in Chapter 9 of CURRENT PROTOCOLS IN

MOLECULAR BIOLOGY, Ausubel *et al.*, *supra* which is incorporated herein by reference.

As used herein, "*derivative*" Cpn 10 proteins of the invention include Cpn10 proteins, which have been altered, for example by conjugation or complexing with other chemical moieties or by post-translational modification techniques as would be understood in the art, inclusive of fusion partner proteins.

Other derivatives contemplated by the invention include, but are not limited to, pegylation, modification to side chains, incorporation of unnatural amino acids and/or their derivatives during protein synthesis and the use of crosslinkers and other methods which impose conformational constraints onCpn10 protein. Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by acylation with acetic anhydride; acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; amidination with methylacetimidate; carbamoylation of amino groups with cyanate; pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$; reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; and trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS).

The carboxyl group may be modified by carbodimide activation via O-acylisourea formation followed by subsequent derivitization, by way of example, to a corresponding amide.

The guanidine group of arginine residues may be modified by formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

Sulphydryl groups may be modified by methods such as performic acid oxidation to cysteic acid; formation of mercurial derivatives using 4-chloromercuriphenylsulphonic acid, 4-chloromercuribenzoate; 2-chloromercuri-4-nitrophenol, phenylmercury chloride, and other mercurials; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; carboxymethylation with iodoacetic acid or iodoacetamide; and carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified, for example, by alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides or by oxidation with N-bromosuccinimide.

Tyrosine residues may be modified by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

The imidazole ring of a histidine residue may be modified by N-carbethoxylation with diethylpyrocarbonate or by alkylation with iodoacetic acid derivatives.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include but are not limited to, use of 4-amino butyric acid, 6-aminohexanoic acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, t-butylglycine, norleucine, norvaline, phenylglycine, ornithine, sarcosine, 2-thienyl alanine and/or D-isomers of amino acids.

Derivatives may also include fusion partners and epitope tags. Well known examples of fusion partners include, but are not limited to, glutathione-S-transferase (GST), Fc portion of human IgG, maltose binding protein (MBP) and hexahistidine (HIS_6), which are particularly useful for isolation of the fusion protein by affinity chromatography. For the purposes of fusion polypeptide purification by affinity chromatography, relevant matrices for affinity chromatography are glutathione-, amylose-, and nickel- or cobalt-conjugated resins respectively. Many such matrices are available in "kit" form, such as the QIAexpress™ system (Qiagen) useful with (HIS_6) fusion partners and the Pharmacia GST purification system.

One particular example of a fusion partner is GST, such as described in Ryan *et al.*, 1995, J. Biol. Chem., **270** 22037-22043. In some cases, the fusion partners also have protease cleavage sites, such as for Factor X_a or Thrombin, which allow the relevant protease to partially digest the fusion polypeptide of the invention and thereby liberate the recombinant Cpn10 protein therefrom. The liberated Cpn 10 protein can then be isolated from the fusion partner by subsequent chromatographic separation. Upon cleavage of GST-Cpn10 the derivative GSM-Cpn10 protein is produced, for example

Fusion partners according to the invention also include within their scope "*epitope tags*", which are usually short peptide sequences for which a specific antibody is available. Well known examples of epitope tags for which specific monoclonal antibodies are readily available include c-myc, haemagglutinin and FLAG tags.

Cpn10 proteins according to the invention (inclusive of fragments,

variants, derivatives and homologues) may be prepared by any suitable procedure known to those of skill in the art, including chemical synthesis and recombinant expression.

Preferably, Cpn10 is recombinant Cpn10.

For example, the recombinant Cpn10 protein may be prepared by a procedure including the steps of:

(i) preparing an expression construct which comprises an isolated nucleic acid encoding Cpn10, operably-linked to one or more regulatory nucleotide sequences in an expression vector;

(ii) transfecting or transforming a suitable host cell with the expression construct; and

(iii) expressing the recombinant protein in said host cell.

The method described in Ryan *et al.*, 1995, *supra* is an example of a recombinant Cpn10 protein production method.

Modulators of cytokine secretion

The present invention provides methods of modulating secretion of immunomodulator, such as pro-inflammatory and anti-inflammatory cytokine and chemokines, by inhibiting, suppressing or otherwise reducing Toll-like and/or β1-integrin signaling. It will also be appreciated that immunomodulator secretion can be modulated by augmenting, strengthening or increasing Toll-like and/or β1-integrin signaling.

The inventors demonstrate that Cpn10 exerts its immunosuppressive effects on immune cells through inhibition of Toll-like and/or β1-integrin

signaling. It is also proposed that the Cpn10 inhibition of Toll-like and/or β1-integrin signaling may be mediated by Hsp60.

Therefore the present invention contemplates use of Cpn10, a protein complex comprising Cpn10 and Hsp60, a protein complex comprising Cpn10, Hsp60 and a Toll-like receptor, and a protein complex comprising Cpn10, Hsp60 and a β1-integrin to produce or design a modulator of immunomodulator secretion.

The modulators of immunomodulator secretion could be agonists, antagonists or mimetics of Cpn10, a protein complex comprising Cpn10 and Hsp60, a protein complex comprising Cpn10, Hsp60 and a Toll-like receptor, and a protein complex comprising Cpn10, Hsp60 and a β1-integrin.

The term *"mimetic"* is used herein to refer to molecules that resemble particular structural and/or functional regions or domains of proteins or peptides, and includes within its scope the terms *"agonist"*, *"analogue"* and *"antagonist"* as are well understood in the art.

An *"agonist"* refers to a molecule, such as a drug, enzyme activator or protein, which enhances activity of another molecule or receptor site.

For example, modulators of immunomodulator secretion may be identified by way of screening libraries of molecules such as synthetic chemical libraries, including combinatorial libraries, by methods such as described in Nestler & Liu, 1998, Comb. Chem. High Throughput Screen. 1, 113 and Kirkpatrick *et al.*, 1999, Comb. Chem. High Throughput Screen 2, 211.

It is also contemplated that libraries of naturally-occurring molecules may be screened by methodology such as reviewed in Kolb, 1998, Prog. Drug. Res. 51, 185.

More rational approaches to designing modulators as described herein may employ computer assisted screening of structural databases, computer-assisted modelling and/or design, or more traditional biophysical techniques which detect molecular binding interactions, as are well known in the art.

Computer-assisted structural database searching, modelling and design is becoming increasingly utilized as a procedure for engineering agonists and antagonist molecules. Examples of database searching methods may be found in International Publication WO 94/18232 (directed to producing HIV antigen mimetics), United States Patent No. 5,752,019 and International Publication WO 97/41526 (directed to identifying EPO mimetics), each of which is incorporated herein by reference.

Generally, other applicable methods include any of a variety of biophysical techniques which identify molecular interactions. Methods applicable to potentially useful techniques such as competitive radioligand binding assays, analytical ultracentrifugation, microcalorimetry, surface plasmon resonance and optical biosensor-based methods are provided in Chapter 20 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997) which is incorporated herein by reference.

Pharmaceutical compositions and methods of treatment

The invention provides use of Cpn10 for targeting Toll-like and/or β1-integrin and Hsp60-mediated immunomodulator secretion. Thus, the present

invention contemplates use of Cpn10 for the therapeutic or prophylactic treatment of diseases, disorders or medical conditions caused by abnormal, excessive or inappropriate cytokine secretion and cell, tissue or organ transplantation, such as septic shock, inflammatory bowel disease, arthritis, psoriasis, congestive heart disease, cachexia, cancer, GVHD and chronic pulmonary disease. The present invention also contemplates use of Cpn10 for the treatment of diseases responsive to inhibition of pro-inflammatory cytokine secretion.

The invention also provides pharmaceutical compositions that comprise Cpn10 or a derivative of Cpn10.

Suitably, the pharmaceutical composition comprises an appropriate pharmaceutically-acceptable carrier, diluent or excipient.

By "*pharmaceutically-acceptable carrier, diluent or excipient*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates and pyrogen-free water.

A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991) which is incorporated herein by reference.

Any safe route of administration may be employed for providing a patient with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the like may be employed. Intra-muscular and subcutaneous injection is appropriate, for example, for administration of immunogenic compositions, vaccines and DNA vaccines.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as is pharmaceutically-effective. The dose administered to a patient, in the context of the present invention, should be sufficient to affect a beneficial response in a patient over an appropriate period of time. The quantity of agent(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof, factors that will depend on the judgement of the practitioner.

In a particularly preferred form, the pharmaceutical composition is a composition suitable for controlled release of Cpn10.

By this is meant a composition which controls and the rate of delivery of Cpn10 over a sustained period and hence improves the bioavailability and/or efficacy of administered Cpn10.

As will be described in more detail hereinafter, sustained release of Cpn10 is superior to subcutaneous administration of Cpn10 in regulating production of TNFα and IL-10 by LPS-stimulated cells.

Non-limiting examples of controlled release formulations and delivery devices include osmotic pumps, polylactide-co-glycolide (PLG) polymer-based microspheres, hydrogel-based polymers, chemically-crosslinked dextran gels such as OctoDEX™ and dex-lactate-HEMA, for example.

Pharmaceutical compositions and methods of treatment according to the invention may be suitable for medical and/or veterinary use and accordingly practiced on human and non-human animals inclusive of mammals such as humans, livestock, domestic animals and performance animals, although without limitation thereto.

So that the present invention may be more readily understood and put into practical effect, the skilled person is referred to the following non-limiting examples.

EXAMPLES

Example 1 – Cpn10 modulation of LPS-stimulated cytokines and chemokines

Materials and Methods

Production and Purification of Cpn10

Frozen recombinant Cpn10 was dissolved in 50 mM Tris buffer pH 7.6, 150 mM NaCl and was stored at -20^{0}C. The purity of Cpn10 was determined to be >97%. Aliquots were thawed once prior to use. All batches of Cpn10 were active in a rhodanese refolding assay and showed the same molar activity as GroES (data not shown).

LPS and endotoxin assays

LPS from *E. coli* (Sigma L-6529. Strain 055:B5) was dissolved in distilled water and stored at 4^{0}C at 1 mg/ml in glass vials. Immediately prior to use the solution was vigorous vortexed for 5 mins before aliquots were taken. In medium 1 ng/ml of LPS corresponded to 0.57 endotoxin units (EU)/ml. LPS contamination of Cpn10 was determined by the Limulus Amebocyte Lysate assay (BioWhitiker) and all batches of Cpn10 contained <1 EU/mg of purified Cpn10 protein.

Cell lines

K562 (human erythroleukemia), Mono Mac 6 (human monocytic line), U937 (human histiocytic lymphoma), P815 (mouse mastocytoma), EL4 (mouse T cell lymphoma), Jurkat (human T cell leukemia), RAW 264.7 (ATCC TIB 71, mouse macrophage), L929 (mouse fibrosarcoma), B16 (mouse melanoma), HeLa (human cervical carcinoma), and MCA-2 (mouse fibrosarcoma) cell lines were shown to be mycoplasma negative. Cells were grown in medium comprising RPMI 1640 (Gibco Labs, Life Technologies, Grand Island, N.Y., USA), 10% fetal calf serum (Life Technologies), 2 mM glutamine (Sigma), 10 mM HEPES (Sigma), 100 ug/ml of streptomycin and 100 IU/ml of penicillin (CSL Ltd,

Melbourne, Australia). The culture medium was determined to have endotoxin levels < 0.01 ng/ml.

RAW264-HIV-LTR-LUC bioassay

RAW264-HIV-LTR-LUC cells were cultured in the presence of G418 (200 ug/ml) for a week after recovery from liquid nitrogen and grown as suspension cultures (Greiner labortechnik, Frickenhausen, Germany). RAW264-HIV-LTR-LUC cells were disaggregated by repeated pipetting and plated at 2.5 x 10^5 cells/ 24 well and incubated overnight (37^0C and 5% CO_2). Cpn10 was then added for 2 h followed by LPS at the indicated concentrations, and after a further 2 h the adherent cells were processed for the luciferase assay (Luciferase Assay System, Promega. Luciferase activity was read for 15 sec on a Turner Designs Luminometer TD 20/20.

RAW264.7 TNF-α and RANTES assays

Cpn10 and LPS at the indicated concentrations were added to RAW264.7 cells that had been seeded at 5 x 10^4 cells/96 well and cultured overnight. After 3 h (RANTES assay) or 2 hours (TNF-α assay) supernatants (150 µl) were collected and analysed in triplicate for RANTES and TNF-α by Duoset ELISA kit (R & D Systems). The optical density (450 nm) of each sample was determined using a microplate reader (Magellan 3, Sunrise - Tecan, Durham, NC).

Cytokine production from splenocytes and macrophages derived from Cpn10 treated animals

C57BL/6 IL-$10^{-/-}$ mice (H-2^b, Ly-5.2^+) were supplied by the Australian National University (Canberra, Australia). The culture medium used throughout was 10% FCS/IMDM (JRH Biosciences, Lenexa, KS), supplemented with 50

units/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid, 0.02 mM ß-mercaptoethanol, and 10 mM HEPES, and cells were cultured at pH 7.75, 37°C and 5% CO_2. C57BL/6 mice (n=3 per group) were treated with subcutaneous injections of Cpn10 (100 ug) or diluent daily for 5 days, peritoneal macrophages were harvested the next day by peritoneal lavage and pooled from individual animals within the treatment group. Cells were plated in triplicate at $2x10^5$/well in the presence of LPS (1 ug/ml). Culture supernatants were collected at 5 h and levels of TNF-α were assessed by ELISA (see below). Results were normalized to production per 10^5 macrophages based on CD11b staining by FACS analysis of input cells. For IL-10 determination splenocytes were harvested from the same animals and pooled as above and cultured in triplicate at 5 x 10^5/well in the absence (not shown) or presence of LPS (10 ug/ml). Culture supernatants were collected at 48 h and levels of IL-10 determined by ELISA (see below).

Cytokine assays for murine cells stimulated in vitro with LPS

The monoclonal antibody pairs used in the TNF-α and IL-10 ELISA assays were purchased from PharMingen (San Diego, CA) and used at concentrations recommended by the manufacturer. Supernatants were diluted in culture medium 1:1 IL-10 and TNF-α. Cytokines were captured by the capture antibody, and detected by the direct biotin-labelled detection antibody. Strepavidin-labelled horse-radish peroxidase (Kirkegaard and Perry laboratories, Gaithersburg, MD) and substrate (Sigmafast OPD) was then used to measure immobilised biotin. Plates were read at 492 nm using the Spectraflour Plus microplate reader (Tecan). Recombinant cytokines (PharMingen) were used as

standards for ELISA assays. Standards were run in duplicate and the sensitivity of the assays was 15 pg/ml for IL-10 and TNF-α.

Human PBMC TNF-α and IL-10 assays

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood from healthy volunteers by buoyant density gradient centrifugation on Ficoll-Hypaque. PBMCs (1.25 x 10^6 cells/ml) were dispensed at 800 µl/ 24 well (Greiner). Cpn10 was then added for 5 h, followed by LPS and after 20 h, supernatants were collected and duplicate samples analysed for TNF-α and IL-10 production with a human TNF-α IL-10 Duoset ELISA kit (R & D Systems).

Mouse serum TNF-α, RANTES and IL-10 assays after LPS injection

Female 8-10 week old BALB/c mice (Animal Resource Centre, Perth, Australia) were placed under a heating lamp for approximately 10 minutes, then restrained and Cpn10 injected i.v. at the specified doses. After 30 mins, 10 µg LPS was injected i.v. using the same protocol. At 1.5 hours post LPS injection, blood was collected by heart puncture into 1 ml clotting accelerator tubes (MiniCollect, Interpath) and stored at 4°C for analysis of serum TNF-α and RANTES using the ELISA kits (R & D Systems). IL-10 production in serum was measured with mouse OptEIA IL-10 specific ELISA (BD Biosciences Pharmingen).

Bone marrow transplantation and graft versus host disease (GVHD)

Female 8 -14 week old C57BL/6 (B6, H-2^b, Ly-5.2^+), B6 $Ptprc^a$ $Ly\text{-}5^a$ (H-2^b, Ly-5.1^+) and B6D2F1 (H-$2^{b/d}$, Ly-5.2^+) mice were purchased from the Australian Research Centre (Perth, Western Australia, Australia). Cpn10 (100 µg

per animal) or control diluent was injected subcutaneously daily for 5 days into donor and recipient animals prior to transplant. Mice were housed in sterilised microisolator cages and received acidified autoclaved water (pH 2.5) and normal food for the first two weeks post-transplantation. Mice were transplanted according to a standard protocol described previously (Hill *et al.*, 1997, Blood, 90, 3204-3213; Hill *et al.*, 1998, J Clin Invest, 102, 115-123). Briefly, on day 1, B6D2F1 mice received 1300 cGy total body irradiation (^{137}Cs source at 108 cGy/min), split into two doses separated by 3 h to minimise gastrointestinal toxicity. Donor bone marrow (5×10^6) and donor nylon wool purified splenic T cells (2×10^6) were resuspended in 0.25 ml of Leibovitz's L-15 media (Gibco BRL, Gaithersburg MD) and were injected intravenously into each recipient. Survival was monitored daily, and GVHD clinical scores measured weekly. The degree of systemic GVHD was assessed by a scoring system which sums changes in five clinical parameters; weight loss, posture (hunching), activity, fur texture and skin integrity (maximum index = 10) (Hill *et al.*, 1997, Blood, 90, 3204-3213; Hill *et al.*, 1998, J Clin Invest, 102, 115-123; Cook *et al.*, 1996, Blood, 88, 3230-3239). Individual mice were ear-tagged and graded from 0 to 2 for each criterion without knowledge of treatment group. Animals with severe clinical GVHD (scores > 6) were sacrificed according to ethical guidelines and the day of death deemed to be the following day.

Statistical Analysis

Statistical analysis was performed using univariate analysis of variance (ANOVA), t test or log rank statistic using SPSS for Windows 11.5.0 (SPSS Inc.).

Wound healing assay

C57 BL/6 mice were anesthetized with halothane and small surgical incisions (~ 0.5cm) were made in the skin between the scapulae under aseptic conditions. Osmotic pumps (ALZET 1007D) with perfusion rate of 0.5 ul/hr were filled with either Cpn10 (2.25 mg/ml) or control buffer. Using a haemostat, a small pocket was formed by spreading connective tissue apart and osmotic pumps were implanted subcutaneously. The skin incisions were closed with sutures.

Results

Inhibition of LPS signaling by Cpn10 using Raw-264-HIV-LTR-LUC indicator cells

To further investigate the role of Cpn10 as an immunosuppressive agent the ability of Cpn10 to inhibit LPS-mediated NF-κB activation was investigated. The RAW-264-HIV-LTR-LUC cells are a mouse macrophage cell line (RAW264.7) stably expressing a luciferase reporter gene with an HIV long terminal repeat promoter, which is highly and rapidly responsive to NF-κB stimulation. These cells provide a sensitive bioassay for analysis of TLR4 signaling pathways in macrophages stimulated with bacterial LPS (Sweet & Hume, 1995, J Inflamm, 45, 126-135). To avoid the use of supra-physiological levels of LPS, a titration range for LPS concentration was established, which represented approximately 80%, 50% and 20% of maximal LPS-stimulated luciferase activity (5, 1 and 0.2 ng, respectively) (data not shown). Preincubation of the reporter cells with 100 ug/ml of Cpn10 for 2 hours was able to inhibit significantly LPS-stimulated luciferase activity by 30-50% at these concentrations of LPS (Fig. 1). Shorter preincubation times provided less reproducible inhibition and preincubation times above 18 hours provided no inhibition (data not shown).

Cpn10-mediated inhibition of TNF-α and RANTES production in LPS-stimulated RAW264.7 cells

To illustrate that Cpn10-mediated inhibition of LPS-induced NF-κB activation translated to reduction in the secretion of pro-inflammatory mediators, the ability of Cpn10 to inhibit LPS-induced production of the pro-inflammatory cytokine TNF-α and the pro-inflammatory chemokine RANTES was investigated. Incubation of Cpn10 with RAW264.7 cells significantly reduced LPS-induced TNF-α and RANTES secretion (Fig. 1B, C). Increasing the Cpn10 concentration did not significantly enhance the reduction of TNF-α secretion from these cells, and increasing the LPS concentration reduced the Cpn10-mediated reduction in TNF-α secretion (data not shown). The reduction in RANTES secretion induced by 5 ng/ml LPS was dose responsive for Cpn10 concentrations of 50-400 υg/ml; lower doses of LPS failed to induce readily detectable levels of RANTES (data not shown).

Cpn10-mediated inhibition of LPS-induced TNF-α was independent of IL-10

To determine the effect of Cpn10 in a more physiological cell population, mice were treated with Cpn10 and their peritoneal macrophages removed and stimulated with LPS *in vitro*. The Cpn10 treatment significantly reduced the LPS-induced secretion of TNF-α from these cells (Fig. 2A), illustrating that Cpn10 mediates similar effects on macrophages treated *in vivo* as seen with RAW264.7 cells treated *in vitro*.

IL-10 is a potent immunosuppressive cytokine able to inhibit TLR4 signaling (Berlato *et al.*, 2002, J Immunol, 168, 6404-6411; Suhrbier & Linn, 2003, Trends Immunol, 24, 165-168), and when splenocytes from Cpn10 treated

animals were stimulated with LPS, significantly increased IL-10 production was observed compared to control animals (Fig. 2B). However, the Cpn10-mediated reduction in LPS-induced TNF-α production (Fig. 2A) did not require IL-10, since similar reductions in TNF-α secretion were observed when peritoneal macrophages from Cpn10 treated IL-$10^{-/-}$ mice were stimulated with LPS *in vitro* (Fig. 2C). Thus, reduced TNF-α secretion and increased IL-10 production appear to be independent consequences of Cpn10 treatment.

Cpn10-treatment of human peripheral blood mononuclear cells (PBMC)

To determine whether Cpn10 is also active in human cells, PBMC from healthy donors were treated with a concentration range of LPS in the presence and absence of Cpn10. Cpn10-mediated an average 45-66% reduction in LPS-induced TNF-α secretion in a series of repeat experiments (Fig. 3A), illustrating that Cpn10 is also able to inhibit cytokine secretion from primary human PBMC. The ability of Cpn10 to inhibit LPS-induced TNF-α secretion was also shown to be dose dependent with increasing concentrations of Cpn10 mediating increasing inhibition (Fig. 3B). However, increasing levels of LPS reduced the ability of Cpn10 to mediate inhibition of LPS-induced TNF-α secretion (Fig. 3B). Cpn10 treatment also increased the level of LPS-induced IL-10 secretion in PBMC in a dose dependent fashion (Fig. 3C). In contrast to the Cpn10-mediated reduction in TNF-α secretion, Cpn10-mediated elevation of IL-10 production increased with increasing doses of LPS (Fig. 3C), again indicating that Cpn10 affects TNF-α and IL-10 independently.

Cpn10-treatment inhibited LPS-induced TNF-α secretion *in vivo*

A modified endotoxemia model was used to determine whether Cpn10 delivered *in vivo* was able to inhibit LPS-induced TNF-α secretion *in vivo*. BALB/c mice were given 100 υg of Cpn10 iv 30 mins before injection of 10 υg of LPS i.v., and blood removed after 1.5 hours. Cpn10 treatment resulted in an average 47.6% reduction in the serum TNF-α, an average 40.1% reduction in serum RANTES, and an average 43.3% increase in serum IL-10 levels in several repeat experiments (Fig. 4A). Five days of daily Cpn10 pretreatment failed significantly to enhance this level of TNF-α inhibition (data not shown). These data are consistent with the previous tissue culture experiments and illustrate the *in vivo* efficacy of Cpn10 in reducing TNF-α and increased IL-10 production after challenge with LPS.

Cpn10 reduced the acute symptoms of Graft verses host disease (GVHD)

Acute GVHD following allogeneic bone marrow transplantation (BMT) is a T cell-mediated disease in which donor T cells recognise recipient allo-antigens and differentiate in a Th1 dominant fashion. The resulting T cell-derived Th1-cytokines (primarily IFN-γ) prime the donor mononuclear cells to release cytopathic quantities of inflammatory cytokines (e.g. TNF-α) when they are stimulated with LPS that has leaked through the radiation-damaged gastrointestinal mucosa. These cytokines and the allo-reactive T cells then contribute to increasing gastrointestinal damage and LPS leakage. GVHD mortality in BMT models is prevented if the donor mononuclear cells lack TLR4, LPS is effectively blocked (by therapeutic antagonists) (Cooke *et al.*, 2001, J Clin Invest, 107, 1581-1589), or TNF-α itself is neutralized. The ability of Cpn10 administration during the peri-transplant' period to ameliorate GVHD was

therefore investigated. Cpn10 treatment of transplant donors and recipients prior to transplant significantly delayed GVHD mortality (Fig. 4B). In addition, the severity of GVHD as determined by clinical score was also reduced early after BMT (Fig. 4B). Although Cpn10 was able to delay GVHD and reduce early morbidity, ultimately the animals succumbed to GVHD, consistent with the inability of Cpn10 to abolish TNF-α secretion or to effect T cell proliferation and IFNγ secretion (Fig. 2). Treatment of animals with Cpn10 post transplant failed to affect significantly GVHD (data not shown).

Cpn10 and wound healing

The wounds of the mice were inspected 5 days after the procedure. In the group of animals treated with Cpn10 improved healing of the wounds was observed demonstrated by completely closed surgical incisions and the absence of surrounding inflammation in comparison to the buffer treated control mice.

Discussion

Cpn10 mediated a 25-70% inhibition of TNF-α secretion depending on the system used and the dose of LPS and Cpn10. Cpn10 increased LPS-induced IL-10 secretion by approximately 30-200% depending on the system, but the inhibition of TNF-α secretion was not dependent on the elevation of IL-10.

Since *E. coli*-derived LPS is a well-described agonist for TLR4, the experiments indicate that Cpn10 modulates TLR4 signaling through the NF-κB pathway. However, it is likely that Cpn10 modulates cytokine secretion via other pathways stimulated by the TLR4 and TLR2 complex.

The inhibitory effects of Cpn10 are mediated very rapidly, within 30 mins (Fig. 4A) to 2 hours (Fig. 1A). This implicates inhibition of early signaling

events or activation of rapid negative feedback mechanisms like PI3K, rather than late phase feedback mechanisms involving IRAK or SOCS (Fukao & Koyasu, 2003, *Trends Immunol,* 24, 358-363).

The specific PI3K inhibitor, wortmannin, had no detectable effect on Cpn10 activity (data not shown), suggesting Cpn10 does not affect the P13K pathway.

Example 2- Cpn10 and Hsp60 in TLR4 signaling

HSP60 and Cpn10 interact in the mitochondria in the presence of ATP and magnesium. Both cofactors are also present in the extracellular fluid and Hsp60 is believed to contribute to TLR4 signalling. Since Cpn10 inhibits LPS-induced TLR4 signalling, Cpn10 may bind and/or remove Hsp60 from the cell surface thereby mediating its negative effect on TLR4 signalling. Cells A549 (adherent human lung adenocarcinoma) cells are known to express detectable Hsp60 on the cell surface (Shin *et al.*, 2003. JBC 278;7607). Hsp60 was detected by Western analysis of cell surface proteins, which were prepared by biotinylating the cell surface and separating the biotinylated proteins using strepavidin beads (Shin et al., 2003). Here we determine whether cell surface HSp60 is removed when cells are treated exogenously with Cpn10.

Materials and Methods

Cpn10

Cpn10 batches were dissolved in Tris saline buffer pH 7.6 and transported frozen on dry ice in aliquots and stored at -20^{0}C. Aliquots were thawed once prior to use.

Inhibition of Cpn10 activity with anti-Hsp60 antiserum

RAW264-HIV-LTR-LUC cells were plated and incubated overnight as above. The medium was replaced and goat polyclonal anti-Hsp60 antiserum (1/1000 dilution) (Stressgen, Victoria, Canada), or the same dilution of normal goat serum was added for 1 hour. Cpn10 was then added for 2 hours, followed by LPS and luciferase activity measured 2 hours later.

Cpn10 treatment, biotinylation and Western analysis

A near confluent T75 flask of A549 cells was treated with culture medium or Cpn10 (100 ug/ml) in culture medium for 2 hours, the cells were then washed 3 times with 25 ml Dulbecco's PBS (with Ca/Mg) (DPBS) pH=8 , at 4°C, for 4-5 mins each. Cell surface proteins were labelled with 5 ml 4 mM EZlink sulpho-NHS-LC-Biotin (Pierce) in DPBS, 37°C, 30 mins, with occasional rocking. The solution was removed and the cells washed once with 50 mM Tris HCl to stop the reaction and then twice more with cold DPBS. Lysis buffer (2 ml of DPBS, 1% NP40, plus protease inhibitor; aprotinin, leupeptin and PMSF) was added, the cells were scrapped from the flask, briefly sonicated and centrifuged (top speed on a bench microfuge) and supernatant retained (all at 4°C). Strepavidin-agarose beads (300 ul) (Sigma) were washed extensively; 4x in PBS supplemented with 1% NP-40). Biotinylated sample (200 ul) was added to the strepavidin bead pellet and made up to 2 ml with PBS/1% NP-40. The mix was gently rotated for 1 h at 4^0C. The beads were then washed 3x 10 ml with PBS/1% NP-40. The agarose pellet was resuspended in 200 ul of PAGE loading buffer (including DTT), boiled for 2 min then cooled on ice, centrifuged (top speed on a bench microfuge), and supernatant loaded onto SDS PAGE gels.

Western blotting used rabbit anti-Hsp60 (Santa Cruz, used at 1/500), goat anti-Hsp60 (Santa Cruz, used at 1/500), mouse anti-actin (Santa Cruz, used at 1/500), and rabbit anti-Cpn10 (supplied by CBio used at 1/100). The Western blot membranes (Hybond-C, Amersham) were stripped with Restore Western Blot Stripping Buffer (Pierce). Markers were Bench Mark Pre-stained protein ladder (Invitrogen). Second antibodies were sheep HRP anti-rabbit IgG, donkey HRP anti-goat IgG, and sheep HRP anti-mouse (Chemicon used at 1/2000). Signal was developed using ECL Western blotting analysis (Amersham) or Super Signal West Pico Chemoluminescent Substrate (Pierce) for anti-actin.

Results

We initially tested whether blocking Cpn10 binding to Hsp60 with anti-Hsp60 antiserum would prevent Cpn10-mediated inhibition of LPS-induced TLR4 signaling. Pre-incubation of RAW264-HIV-LTR-LUC cells with anti-Hsp60 serum, but not control serum, prevented the Hsp10-mediated reduction of LPS-induced NF-κB activation (Fig. 5). Furthermore, anti-Hsp60 antibody mediated a similar level of inhibition of LPS-signaling as was seen for Hsp10 treatment (Fig. 5). (The lack of detectable LUC activity when cells were incubated with either antisera alone, illustrated that endotoxin contamination of the antibodies was below the level of detection).

Western analysis of streptavidin purified biotinylated cell surface proteins from control and Cpn10 treated using anti-actin antibody showed no detectable bands unless the membrane was substantially over-exposed (30 min exposure), even then only very faint bands were detected below the 48.8 Kd marker were actin usually runs (Fig. 6 A). Other non-specific bands were substantially more

prominent for this over-exposure, particularly the dense bands between the 6 and 14.8 Kd markers, which can also be seen in FIG. 6B and 6C. The anti-actin analysis was performed on the same membrane as shown in Fig. 6 B after the membrane had been stripped of antibody. Thus the biotinylation reaction did not result in significant biotinylation of intracellular proteins, since very little of the abundant cytoplasmic protein, actin was biotinylated.

Western analysis using anti-Cpn10 revealed no differences in the presence of Cpn10 reactive bands between Cpn10 and control treated A549 cells, indicating that exposure to Cpn10 did not cause detectable binding of Cpn10 to the cell surface. The most prominent band ran just above the 25.9 Kd marker and appeared equally abundant in both Cpn10 and control treated A549 cells.

Western analysis using anti-Hsp60 illustrated that Hsp60 can indeed be detected on the cell surface using the biotinylation methodology. Although Hsp60 was not removed its abundance did drop by ≈27% as assessed by scanning densitometry of lanes 3 and 4 (Fig. 6D), taking account background in Fig. 6 D, and assuming equal protein loading based on similar intensity bands in Fig. 6C. It should be noted that Western analysis is semi-quantitative.

Conclusion

These data suggest that Hsp60 may be involved in the Hsp10-mediated reduction of LPS-signaling. However, it is also possible that anti-Hsp60 and Hsp10 independently modulate TLR4 signaling in a non-additive manner.

Evidence is also provided that Cpn10 reduces cell surface Hsp60 levels by 27%. Longer exposure (ie 24 h rather than 2 h) of the cells to Cpn10 may be required to increase the level of Hsp60 depletion. Increasing the levels of ATP

and Mg may also facilitate removal of Hsp60, as these compounds facilitate Cpn10 interaction with Hsp60.

Perhaps surprisingly no change in anti-Cpn10 reactive bands could be seen after treatment with Cpn10. Thus these experiments do not provide evidence for stable cell surface binding of Cpn10 under these conditions.

Example 3– Cpn10 and TLR2

Methods

BALB/C mice were injected subcutaneously with of ovalbumin (10 ug) (Sigma) emulsified in Complete Freund's adjuvant (CFA) (Sigma). CFA contains mycobacterial cell wall extracts which are believed to contain lipopeptide agonists of TLR2 (Lim *et al.*, 2003, Int Immunopharmacol, 3(1): 115-118; Tsuji *et al.*, 2000, Infect Immun; 68, 6883-6890; Kirschning & Schumann, 2002, Curr Top Microbiol Immunol, 270, 121-44). CFA is well known to induce granulomas (Bergeron *et al.*, 2001, Eur Respir J, 18(2), 357-361; Shah *et al.* 2001, J Assoc Physicians India, 49, 366-368).

Cpn10 (100 ug) was given twice daily for 5 days with two doses preceding injection of the CFA. Subcutaneous granulomas were measured at the indicated times.

Results

To determine whether Cpn10 is able to infect CFA's granuloma formation activity, Cpn10 treated and buffer control treated mice where injected with CFA. Cpn10 treatment significantly reduced the size of the granuloma induction (Fig. 7A).

Conclusion

As CFA stimulates TLR2 and CFA induces granuloma formation, a reduction in granuloma formation mediated by Cpn10 treatment provides evidence that Cpn10 also inhibits TLR2 signaling.

Example 4-Cpn10 inhibits activation of NF-κB by the TLR2 agonist $PAM_3CYS\text{-}SK_4$

$PAM3CysSK_4$ (a lipopeptide) is a known agonist of TLR2 (Agrawal *et al.*, J Immunol, 2003, 171, 4984-9) and is able to stimulate the HIV LTR (Fig. 7B) which is thought to activate transcription factors, such as NF-κB (Lee *et al.*, J Immunol. 2002. 168(8):4012-4017).

Material and Methods

PAM_3CysSK_4 was purchased from EMC Microcollection GmbH and dissolved as a working stock dilution of 1 mg/ml in water. Cpn10 and the RAW 264 -HIV LTR Luc assay system was performed as described previously for LPS. Briefly Raw-luc cells were seeded at 2.5 x 10^5 cell/ml into 24 well plates and incubated overnight at 37 C. Cpn10 was added to the cells at 120 υg/ml and incubated for 2h at 37^0C. $PAM_3Cys\text{-}SK_4$ (10 ng/ml or 2 ng/ml or 0 ng/ml) was then added for 2 h prior to the luiferase assay. LPS at 1 ng/ml or 0.2 ng/ml was used as a positive control.

Results

Cpn10 inhibited HIV LTR activation by the TLR2 agonist $PAM3CysSK_4$ (Fig. 7B). This inhibition was maintained irrespective of whether the medium was changed before addition of Cpn10 (Fig. 7C) or before addition of $PAM3CysSK_4$ (Fig. 7D).

Conclusion

Cpn10 is able to inhibit pro-inflammatory mediator activation signals in macrophages stimulated by a TLR2 agonist.

Example 5 – Cpn10 and TLR3

Methods

RAW264-HIV-LTR-LUC cells were stimulated with poly IC (synthetic double stranded RNA) using the same methods described above for LPS stimulation. Double stranded RNA is an agonist of TLR3 and stimulates NF-κB (Suhrbier & Linn, 2003, Trends Immunol, 24(4), 165-168).

Results

To determine whether Cpn10 was able to inhibit polyIC-induced TLR3 signaling, RAW264-HIV-LTR-LUC cells were treated with 100 ug/ml of Cpn10 for 2 h followed by polyIC for 2 h before analysis of the LUC (luciferase) levels was undertaken. The % inhibition of the RLU (relative light unit) is shown (Fig. 8). As a positive control inhibition of LPS/TLR4 inhibition was conducted in parallel control.

Conclusion

As polyIC is a known TLR3 agonist these data indicate that Cpn10 is able to inhibit TLR3 signaling.

Example 6 – Cpn10 and cachexia

Methods

Induction of Adjuvant-induced cachexia

The aim of the present study was to determine whether Cpn10 administration to rats during development of adjuvant arthritis would result in decreased weight loss. Female Dark Agouti rats (n=30, 150-160 g) were injected

subcutaneously with 0.1 ml of Complete Freund's adjuvant (CFA) at the base of the tail. The adjuvant consisted of incomplete Freund's adjuvant (Difco, Michigan, USA) to which was added 10 mg/ml heat-killed *Mycobacterium tuberculosis* H37RA (Difco). The onset of detectable arthritic disease in this model is generally 8-10 days after CFA injection.

Cpn10 Treatment

Rats were injected subcutaneously with 0.25 mg/kg (n=10) or 2.5 mg/kg Cpn10 (n=10) or diluent control (Tris/saline buffer) (n=10) daily from day 2 to day 13, and weighed on a daily basis.

Statistical Analysis

The difference in weight observed in rats receiving Cpn10 or diluent control were tested for significance using univariate analysis of variance (ANOVA).

Results

There was a net loss of weight in all groups following administration of CFA, which appeared more marked in the control group (Fig. 9).

When the weight loss data for the 0.25 and 2.5 mg/kg Cpn10 treatment groups were pooled and compared with the control group, there was a statistically significant difference (p=0.027) in weight loss. The pooling of data from the two treatment groups is justified in this case due to the similar values of weight loss in the two groups (p=0.94) over this time period.

Conclusion

Adjuvant arthritis leads to changes in body composition and cytokine production that mimics pro-inflammatory cytokine-driven cachexia in chronic

inflammatory arthritis (Mayer, 1997, Arthritis Rheum, 40, 534-539). Cpn10 administration *in vivo* or *in vitro* reduces production of pro-inflammatory cytokines by cells stimulated by LPS and other agonists.

The effects of Cpn10 were tested at two doses in rats in which cachexia was induced experimentally with a single injection of CFA. Diluent control and Cpn10 were administered subcutaneously to animals of similar weight and age. The administration of CFA resulted in a significant decrease in body weight. By comparing the Cpn10 treated versus control treated groups, there was a statistically significant reduction in weight loss in the Cpn10-treated rats.

Elevated levels of inflammatory cytokines including TNF-α, IL-1β and IL-6 are known to correlate with cachexia in a number of diseases, including cancer and rheumatoid arthritis (Argiles, 2003. Curr Opin Clin Nutr Metab Care, 6(4); 401-406; Walsmith, 2002, Int J Cardiol, 85, 89-99). We have shown that administration of Cpn10 reduces production of TNF-α and RANTES, and increases production of the anti-inflammatory cytokine IL-10 in murine models of endotoxemia and graft-versus-host-disease, and *in vitro* LPS-stimulation of freshly isolated PBMC and monocyte cell lines.

Example 7- Cpn10 inhibits LPS-induced TNF-α and IL-6 secretion

Materials and Methods

TNF-α and IL-6 Assays

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood from healthy volunteers by buoyant density gradient centrifugation on Ficoll-Paque Plus (Amersham, Uppsala, Sweden). PBMCs (1.25×10^6 cells/ml) were dispensed at a final density of 1×10^6 viable cells/ml in

24-well tissue culture plates (Greiner Bio-One, Kremsmuenster, Austria). Cpn10 was then added and plates were incubated for 5 h, followed by LPS addition and a further 20 h incubation, after which supernates were collected and duplicate samples analyzed for TNF-α and IL-6 levels (Duoset ELISA kits, R & D Systems).

Results

To determine whether Cpn10 is active on primary human cells, PBMC from healthy donors were pre-treated with Cpn10 or buffer for 1 h and then stimulated with 0.04 ng/ml LPS. This dose of LPS was established as the lowest dose reliably able to stimulate significant TNF-α secretion and corresponds to the dose in humans that produces a mild transient syndrome similar to clinical sepsis Lynn et al., 2003, J. Infect. Dis. **187**, 631-639

In PBMC from 5 donors, 1 μg/ml Cpn10 mediated an average 24.8 % reduction, and 10 ug/ml an average 30.4% reduction in LPS-induced TNF-α secretion (Fig. 10*A*), illustrating that Cpn10 also reduces LPS-induced TNF-α secretion from PBMC. To illustrate that tolerance induction was not operating in this system, PBMC from 3 donors (1, 2 and 3) were pre-treated with a range of LPS concentrations and 1 h later were stimulated with 0.04 ng/ml LPS. As seen in Fig. 10*B*, LPS pretreatment for 1 h did not inhibit TNF-α secretion stimulated by the second LPS treatment.

To determine whether Cpn10 inhibited LPS-induced IL-6 secretion, PBMC from eight donors were treated with 10 ug/ml Cpn10 or buffer for 1 h followed by stimulation with 0.04 ng/ml LPS. An average 24.4 % reduction in

LPS-induced IL-6 secretion was observed (Fig. 10C). LPS pretreatment for 1 h did not inhibit IL-6 secretion stimulated by a second LPS treatment, illustrating that tolerance induction was not operating in this system (Fig. 10D).

Example 8-Cpn10 does not induce tolerance

Materials and Methods

RAW264-HIV-LTR-LUC Bioassay

RAW264-HIV-LTR-LUC cells were cultured in the presence of G418 (200 μg/ml) for one week after recovery from liquid nitrogen and grown as suspension cultures (Greiner Labortechnik, Frickenhausen, Germany). RAW264-HIV-LTR-LUC cells were disaggregated by repeated pipetting and plated at 2.5x 10^5 cells/well in 24-well plates and incubated overnight (37^0C and 5% CO_2). LPS from *E. coli* (Sigma L-6529. Strain 055:B5, Sigma, St Louis, MO) was dissolved in sterile distilled water and stored at 4^0C at 1 mg/ml in glass vials. Immediately prior to use the solution was vigorously vortexed for 5 mins before aliquots were taken. The cells were incubated with LPS, Cpn10 or control buffers for 2 h, followed by the addition of stimulating LPS at the indicated concentrations. After a further 2 h incubation, the adherent cells were processed for the luciferase assay (Luciferase Assay System, Promega, Madison, WI). Luciferase activity was read for 15 sec on a Turner Designs Luminometer TD 20/20.

Production and Purification of Cpn10

Recombinant human Cpn10 (GenBank accession no. X75821) was produced in *E. Coli* essentially as described by Ryan *et al*, *supra*. In addition, the material that did not bind Macro-Prep High Q (BioRad) was further purified by S-Sepharose and then Gel-Filtration (Superdex 200, Amersham Biosciences).

Purified Cpn10 in a 50 mM Tris-HCl (pH 7.6) and 150 mM NaCl buffer, was filtered through an Acrodisc with a 0.2 mm Mustang E membrane according to the manufacturer's instructions (Pall Corporation, Ann Arbor, MI. Cat. No. MSTG5E3) to remove residual endotoxins and was stored at -70^{0}C. The purity of Cpn10 was determined to be >97% by SDS-PAGE. Aliquots were thawed once prior to use. All batches of Cpn10 showed the same molar activity as GroES in GroEL-mediated rhodanese refolding assays (Brinker et al., 2001 *Cell* 107, 223-233; data not shown). LPS contamination of Cpn10 was determined by the Limulus Amebocyte Lysate assay (BioWhittaker, Walkersville, MD) and all batches contained <1 EU/mg of purified Cpn10 protein.

Trypsin treatment of Cpn10

2.5% trypsin (Gibco) was filtered through an Acrodisc as above twice and added at 40 ug/ml to Cpn10 (at 2-3 mg/ml). After incubation at 37^{0}C overnight the trypsin/Cpn10 solution was heated to 90^{0}C for 15 mins to destroy trypsin activity prior to addition to the bioassays. After trypsin treatment no Cpn10 could be detected by SDS PAGE and the material was inactive in the rhodenese refolding assay (data not shown).

RAW264.7 RANTES Assays - RAW264.7 RANTES Assays

Cpn10 and LPS at the indicated concentrations were added to RAW264.7 cells that had been seeded at 5 x 10^4 cells/ well in 96-well plates and cultured overnight. After 6 h culture supernates (200 µl) were collected and analyzed in triplicate after dilution (1:4) in PBS supplemented with 1% BSA (pH 7.2 –7.4) using a RANTES ELISA paired antibody kit (R & D Systems, Minneapolis, MN). The optical density (450 nm) of each sample was determined using a microplate

reader (Tecan Sunrise, Austria, with Magellan V3.11 software). The limit of detection for the RANTES ELISA was 31 pg/ml.

Statistical Analysis - Statistical analysis (univariate analysis of variance - ANOVA, Student's *t* test or log rank statistic) was performed using SPSS for Windows 11.5.0 (SPSS Inc.).

Results

Tolerance Induction was Not Responsible for Inhibition of LPS Signaling in RAW-264-HIV-LTR-LUC Cells

LPS tolerance is a well-recognized phenomenon whereby the response to a second stimulus with LPS is reduced. LPS tolerance is normally induced if the time interval between the two LPS exposures exceeds 3 h and the concentration of the LPS during the initial exposure is sufficiently high to stimulate the macrophages (West & Heagy, 2002, *Crit Care Med* 30, S64-S73; Fujihara et al., 2003, *Pharmacol. Ther.* 100, 171-194).

To formally discount tolerance induction as being responsible for the observations in Fig. 11*A*, RAW-264-HIV-LTR-LUC cells were pre-treated with a range of LPS concentrations for 2 h followed by stimulation with 5, 1 and 0.2 ng/ml LPS. Pre-treatment with LPS concentrations ranging from 1 to 0.0005 ng/ml did not inhibit the induction of LUC activity by the second LPS exposure (Fig. 11*C*). Thus for the RAW-264-HIV-LTR-LUC system the 2 h pre-treatment period appeared insufficient for tolerance induction. Furthermore, sub-stimulating doses (0.005-0.0005 ng/ml), potentially similar to those found in Cpn10 preparations, also failed to inhibit LPS-mediated LUC activity. LPS tolerance was therefore unlikely to be responsible for the observations in Fig. 11*A*

since (i) the 2 h interval between LPS exposures was insufficient for LPS tolerance induction in the RAW-264-HIV-LTR-LUC system, and (ii) undetectable (or sub-stimulating) levels of LPS, which are potentially contaminating the Cpn10 preparations, were unable to mediate LPS tolerance.

Cpn10-mediated Reduction of LPS-stimulated LUC Activity in RAW-264-HIV-LTR-LUC Cells was Dose Responsive

The percent reductions in LUC activity shown in Fig. 11*A* were obtained using 100 ug/ml of Cpn10. To determine whether the activity of Cpn10 was dose responsive, RAW-264-HIV-LTR-LUC were treated with a range of Cpn10 concentrations prior to the addition of LPS. (Treatment with 100 ug/ml thus represents a repeat of the experiments shown in Fig. 11*A*). A clear dose response emerged with increasing levels of inhibition apparent from 2 to 100 ug of Cpn10, with the inhibitory appearing to level off after 100 ug/ml.

Cpn10-mediated Reduction of LPS-stimulated RANTES Production by RAW264.7 Cells

To determine whether the Cpn10-mediated reduction in NF-κB activation in RAW264.7 seen in Fig. 11*A* translated to a reduction in LPS-induced RANTES levels, RAW264.7 were treated LPS and a range of Cpn-10 concentrations. The LPS and the Cpn10 were added at the same time to the cells thereby avoiding any tolerance issues. Cpn10 was able to reduce secretion of the chemokine RANTES by 20-30% at 10 ng/ml of Cpn10 (Fig. 11*E*), a concentration of Cpn10 over a 1000 fold lower than that required to inhibit NF-κB stimulation (Fig. 11*D*). RANTES secretion requires persistent NF-κB stimulation, which may make its

production more susceptible to agents that inhibit NF-κB stimulation (Ting & Endy, 2002, *Science* 298, 1189-1190).

Example 9 – Effect of Cpn10 in human clinical trial subjects

Phase Ia Clinical Trial

Materials and Methods

Nineteen healthy normal volunteers aged between 18 and 55 years were enrolled in a 14-day phase I trial of Cpn10 to assess the pharmacokinetics and safety of Cpn10 administered as a single intravenous infusion or subcutaneous injection in a double-blind placebo control protocol. Following screening and written informed consent, subjects were fasted overnight prior to dosing with Cpn10 at 1, 2.5, 5 or 10 mg given as a 10 minute intravenous infusion, or 5 mg given subcutaneously. Blood samples (50 ml) for PBMC isolation were collected prior to dose (approximately 12 hours pre-dose), 8 hours post-dose, and on day 6 following Cpn10 dose. Subjects were monitored for 14 days post treatment for adverse events, with blood drawn at intervals for standard haematology and biochemistry assessment and development of anti-Cpn10 antibodies.

PBMC isolation and storage

PBMC were isolated from heparinized blood by density gradient centrifugation on Ficoll-Hypaque Plus (Amersham) using the manufacturer's protocol. Following two wash steps, cells were resuspended in freezing medium (10% DMSO in foetal bovine serum [FBS]) and frozen using a step-down freezing method at –70°C. Cells were transported on dry ice and then stored in liquid nitrogen until use.

PBMC stimulation

PBMC were thawed and centrifuged through FBS followed by washing and resuspending in RPMI with 10% FBS. Cells were aliquotted at a final density of 1 x 10^6 viable cells/ml in 24-well tissue culture plates with or without LPS. Following 20 hrs incubation at 37°C with 5% CO_2, cell culture supernates were collected and tested for levels of TNF-α using a commercial human TNF-α ELISA (R&D Systems).

Results

We have compared the LPS-driven response at day 1 vs. day 0 (i.e. post-Cpn10 vs. pre-Cpn10) in groups of volunteers given 1 (n=1), 2.5 (n=3), 5 (n=3), and 10 mg Cpn10 (n=3) or placebo (n=3). (Note: PBMC from subjects 001 and 004 (1 mg Cpn10 cohort) were not viable due to haemolysis of blood samples. Therefore, data from PBMC for this cohort includes only one subject.) Figures 12A and 12B demonstrate a dose-responsive Cpn10-mediated change in TNF-α production by PBMC stimulated with a range of LPS concentrations *in vitro*. That is, the LPS-driven response at day 1 relative to day 0 was reduced in 1 out of 3 subjects in the 2.5 mg cohort, in 3 out of 3 subjects in the 5 mg cohort, and in 1 out of 3 subjects in the 10 mg cohort. While inconclusive due to the small cohort size, the data seem to indicate a slight reversal of the trend seen in the 5 mg group, when the dose was increased to 10 mg. In subjects given 5 mg Cpn10 via subcutaneous injection, there does not appear to be a Cpn10-mediated effect on TNF-α production (Figure 12C), however the cohort size is again too small to provide conclusive data.

Conclusions

As a predictor of a Cpn10-mediated change in immune activity during a phase I clinical trial, we collected peripheral blood mononuclear cells (PBMC) approximately 12 hours before, and 8 hours after a single intravenous infusion or subcutaneous injection of Cpn10 (or placebo). These cells were stimulated with a range of LPS concentrations *in vitro* in the absence of exogenous Cpn10 to assess the level of TNF-α production. The data suggest there may be an effect of Cpn10 in reducing the pro-inflammatory response, when administered in the dose range of 2.5 to 10 mg. However, the cohorts in this study were very small and more data will be accumulated to support a hypothesis about Cpn10's biological effect *in vivo*. Data from the 10 mg dose cohort seem to be inconsistent with the trend in TNF-α response from the groups given 2.5 and 5 mg Cpn10, although the small cohort size prohibits any analysis of these data beyond speculation.

The lack of an effect in this *in vitro* assay from PBMC isolated from subjects given 5 mg Cpn10 subcutaneously is most likely related to the relatively small amount of protein that is detectable (and thus bioavailable) in the circulation by ELISA.

We note that PBMC were isolated at a time-point (8 hrs post-dose) at which we could no longer measure Cpn10 in the serum (see PK data), supporting a view that while the $t_{1/2}$ of this recombinant protein is short (~1 hr), its biological effects may be longer-lived. It is also important to point out that the change (i.e. increase) in the LPS response on day 1 vs. day 0 (e.g. in placebo subjects) is a well-documented phenomenon in both rodents and humans, and is thought to be related to the stress response (Granowitz EV *et al.*,1993. J Immunol **151** 1637). That is, the increased LPS-driven response by PBMC on day 1 vs. day 0 may be

due to the stress of the trial, i.e. administration an untested drug, multiple blood draws, etc. Cpn10 (a member of the stress protein group) may be involved in reducing the stress-related exacerbation in the inflammatory response.

Phase 1b Clinical trial

Materials and Methods

Ten volunteers aged between 18 and 65 years with multiple sclerosis not currently receiving immunomodulatory treatment were enrolled in a 28-day phase Ib trial to assess the safety, tolerability and pharmacokinetics of Cpn10 administered as multiple intravenous infusions. This was a placebo-controlled double blind multiple dose escalation study. Similar to the design of the Phase Ia, subjects were dosed with Cpn10 (or placebo) at 5 or 10 mg in an intravenous infusion. Blood samples for PBMC isolation were collected approximately 12 hours pre-dose and 8 hours post-dose on days 1 and 5. To assess of the longevity of the biological effect of Cpn10, blood was drawn for PBMC isolation at 3 and 7 days following the last infusion of the compound (at day 5).

PBMC were isolated, stored and stimulated as described for the phase 1a clinical trial.

Results

We have compared the LPS-driven response at days 1, 4, 5, 8, and 12 vs. day 0 (i.e. post-Cpn10 vs. pre-Cpn10) in groups of volunteers given 5 daily intravenous infusions of 2.5 (n=4) or 5 mg Cpn10 (n=4) or placebo (n=2). Figure 13 demonstrates a dose-responsive Cpn10-mediated change in TNF-α production by PBMC stimulated with LPS *in vitro*. Note that data are described as the percent difference in TNF-α produced in response to LPS stimulation at D1

relative to D0, and at D4 relative to D0, etc. That is, the LPS-driven response at D1 relative to D0 was reduced in all 4 subjects in the 5 mg Cpn10-treated cohort by 38-94%. Comparing the D4 values to the D0 TNF-α values within this cohort, the percent reduction in TNF-α production was 36-72%, and 38-59% when comparing the D5 to D0 TNF-α production. There was no reduction in LPS-stimulated TNF-α in the 2.5 mg Cpn10 treatment group at any time-point while 'on drug', i.e. at days 1, 4, and 5 during the trial.

Conclusion

As a predictor of a Cpn10-mediated change in immune activity during a phase Ib clinical trial in volunteers with multiple sclerosis, we collected peripheral blood mononuclear cells (PBMC) approximately 12 hours before, and about 8 hours after intravenous infusion of Cpn10 (or placebo) on days 1, 4 and 5 during a five-day daily infusion protocol. In addition, PBMC were isolated on days 3 and 7 following the final infusion of Cpn10. These cells were stimulated with LPS *in vitro* in the absence of exogenous Cpn10 to assess the level of TNF-α production. The data suggest there may be an effect of Cpn10 in reducing the pro-inflammatory response, when administered at 5 mg/day. That is, in all four subjects in Cohort B given 5 mg/day Cpn10, there was a marked reduction in LPS-stimulated TNF-α on days 1, 4 and 5. By contrast, in subjects given 2.5 mg/day Cpn10, there was no noted reduction in LPS-induced TNF-α production while 'on drug', i.e. on days 1, 4, and 5. There was also no reduction in LPS-stimulated TNF-α production in cells from the two subjects infused with placebo (Cpn10 vehicle). However, the two cohorts in this study were very small and

more data will be accumulated to support a hypothesis about Cpn10's biological effect *in vivo*.

As in the Phase Ia trial, we note that PBMC were isolated at a time-point (8 hrs post-dose) at which we could no longer measure Cpn10 in the serum, supporting a view that while the $t_{1/2}$ of this recombinant protein is short (~1 hr), its biological effects may be longer-lived. However, from these few data points it is impossible to ascribe a longer-lasting effect of Cpn10 on inflammatory cytokine production than 12 hours.

As also noted during the Phase Ia trial, it is also important to point out that the change (i.e. increase) in the LPS response on day 1 vs. day 0 (e.g. in placebo subjects) is a well-documented phenomenon in both rodents and humans, and is thought to be related to the stress response (Granowitz *et al.*,1993, *supra*).

TNF-α production by cells isolated on days 8 and 12 from placebo subjects, as well as some subjects given Cpn10, may be related to the fact that subjects were out-patients at these time-points and therefore may have been less nervous or stressed. As previously suggested, Cpn10 (a member of the stress protein group) may be involved in reducing the stress-related exacerbation in the inflammatory response.

Example 10 – Effect of controlled release of Cpn10 on GVHD

We have previously shown that subcutaneous (SC) administration of Cpn10 (100 μg daily, for 5 days) reduced the acute symptoms of graft versus host disease (GVHD), augmented LPS-induced production of IL-10 and inhibited LPS-induced TNFα secretion in vivo that was independent of IL-10. However, we were not able to demonstrate the effect of Cpn10 on T lymphocyte function

that was previously reported (Morton, 1998, Immunology and Cell Biology, 76: 483-496). We speculated that the short plasma half-life of the molecule after SC administration could be responsible for this discrepancy. Therefore we next determined if Cpn-10 administered by continuous infusion via osmotic pumps (OP) would affect T cell function and / or augment the observed effect of SC Cpn10 on LPS-induced TNFα and IL-10 production.

Materials and Methods

Osmotic pump implantation

C57Bl/6 mice were anesthetized with isoflurane and small surgical incisions were made in the skin between scapulae under aseptic conditions. Using a hemostat a small pocket was formed by spreading the connective tissue apart and osmotic pumps (Alzet 1007D, perfusion rate 0.5 μl/h) field with Cpn10 (2.25 mg/ml) (n=3 per group) or control buffer (n=3) were implanted subcutaneously. The skin incisions were closed with surgical sutures. Animals were caged individually and sacrificed 5 days later.

Subcutaneous administration of Cpn-10

In order to compare effects of OP Cpn10 to SC Cpn10, control C57/Bl/6 mice were injected for 5 days with Cpn-10 (2.25mg/ml)(n=3) or control buffer (n=3) at the daily dose that was equivalent to the dose received by infusion (27 μg/ day).

Proliferation assay and mitogen stimulation from mixed lymphocyte cultures

For T cell proliferation assays, nylon wool purified T cells from Cpn10 or control-treated C57Bl/6 mice were pooled within the treatment groups and cultured in triplicate (10^5 $CD3^+$ cells/96 well, based on the CD3 staining of the

input cells) with 5 x 10^4 irradiated (2000cGy) B6D2F1 peritoneal macrophages in mixed lymphocyte cultures (MLC). Cultures were pulsed with ^{3}H-thymidine (1 μCi/well) at 72h, cells harvested 16 hours later and tritium decay measured on a 1205 Betaplate reader (primary MLC). To determine secondary T cell proliferative response to allo-antigens and mitogens, spleen derived nylon wool purified T cells (3x10^6 CD3 positive cells/24 well) from Cpn-10 treated or control treated C57Bl/6 mice were stimulated in vitro with irradiated (2000cGy) allogeneic B6D2F1 splenocytes (5x10^6/24 well). After 7 days cells were collected and plated in triplicate (10^5 CD3 positive cells/96 well) and re-stimulated with plate-bound antibodies to CD3 and CD28 (from hybridomas 2C11 and N37.51respectivly) and concanavalin A (ConA);2.5 μg/ml). Cultures were pulsed with ^{3}H-thymidine (1 μCi/well) at 24h, cells harvested 16 hours later and tritium decay measured on a 1205 Betaplate reader (secondary MLC).

Cytokine production from macrophages and splenocytes

C57Bl/6 mice treated for 5 days with Cpn10 or control buffer (OP or SC) were sacrificed on day 6. Peritoneal macrophages were harvested by peritoneal lavage and pooled from individual animals within the treatment group. Cell were plated in triplicate at 2x10^5/well in the presence of LPS (1 μg/ml). Culture supernatants were collected at 5h and levels of TNFα were assessed by ELISA. Results were normalized to production per 10^5 macrophages based on CD11b staining by FACS analysis of input cells. For IL-10 determination splenocytes were harvested from C57Bl/6 animals and pooled as above and cultured in triplicate at 5x10^5/well in the presence of LPS (10 μg/ml). Culture supernatants

were collected at 48 h and levels of IL-10 determined by ELISA.

Results

We first examined the effect of OP Cpn10 on T cell function and compared it to the effect of SC Cpn10. Percentage of change (reduction or increase) induced by OP or SC Cpn10 was calculated compared to the corresponding buffer controls. As shown in Figure 14, OP Cpn10 induced ~50% reduction while SC Cpn10 induced ~30% increase of T cell proliferation in primary MLCs (1°MLC). In addition, secondary T cells response (2° MLC) to alloantigen was reduced by OP Cpn10 (~70%) while SC Cpn10 induced ~55% reduction. Furthermore, T cell proliferative response to stimulation with plate-bound anti-CD3/CD28 antibody was consistently reduced by OP Cpn10 (~60%) while SC Cpn10 had completely opposite effect (~870% increase). Similarly, proliferative response of T cells to Con A was reduced by OP Cpn10 by ~56%, while SC Cpn10 induced ~50% increase of T cell proliferation. There was no difference in the levels of INFγ, IL-4 and IL-10 in the culture supernatants obtained from MLCs (data not shown). Consistently with our previous results with 100 μg/day SC Cpn10, much lower dose of Cpn10 (27μg/day) delivered via OP significantly impaired LPS-induced production of TNFα from peritoneal macrophages (83% reduction) and enhanced IL-10 production from splenocytes (26% increase). On the contrary, SC Cpn10 given at the same daily dose (27μg/day) had just mild effect on TNFα production (~7% decrease) and had opposing effect on LPS-induced production of IL-10 (~16% decrease).

Thus, continuous infusion of Cpn10 via OP induces impairment of T cell proliferation and is superior to SC Cpn10 in regulating production of TNFα and IL-10 by LPS stimulated cells.

SUMMARY

In addition to its critical role in protein folding within the mitochondria, Cpn10 appears to have an extracellular role in the modulation of specific inflammatory processes. In a number of different human and murine *in vitro* systems and in two murine disease models, Cpn10 consistently inhibited LPS-induced secretion of the pro-inflammatory cytokines TNF-α and IL-6 and/or the pro-inflammatory chemokine RANTES, and increased LPS-induced secretion of the anti-inflammatory cytokine IL-10. The Cpn10-mediated reduction in TNF-α secretion was never absolute; instead Cpn10 mediated a 25-70% reduction in TNF-α levels, depending on the system and the dose of LPS and Cpn10. Cpn10 increased LPS-induced IL-10 secretion by approximately 30-200% depending on the system, but the reduction in TNF-α secretion was not dependent on this elevation of IL-10.

Given that *E. coli*-derived LPS is a well-described agonist for TLR4, the experiments described herein indicate that Cpn10 can down-modulate TLR4 signaling. How exactly Cpn10 mediates its inhibitory activity is unclear although it appears to effect inhibition very rapidly, within 30 mins (Fig. 5*A*) to 2 h (Fig. 11*A*). This might implicate inhibition of early signaling events or activation of rapid negative feedback mechanisms such as phosphoinositide 3-kinase (PI3K). However, we have been unable to prevent Cpn10 activity with the specific PI3K

inhibitor, wortmannin, suggesting this pathway is not involved in the Cpn10 mechanism of action.

Cpn10 might inhibit LPS-induced TLR4 signaling by binding to Cpn60 in the extracellular milieu. This notion was supported by the ability to block Cpn10 activity with anti-Hsp60 antibody (Fig. 5). Anti-Hsp60 antibodies by themselves were also able to inhibit LPS-mediated signaling. Interestingly, anti-Hsp60 antibodies and Cpn10 appeared to inhibit LPS-mediated activation with similar efficiency (Fig. 5), perhaps suggesting that both anti-Hsp60 antibodies and Cpn10 bind Hsp60 and prevent Hsp60-mediated augmentation of LPS signaling. We have been unable to activate RAW264.7 cells with recombinant Hsp60 that is substantially free of LPS contamination , suggesting that Hsp60 alone may not be sufficient to stimulate TLR4 signaling. Instead Hsp60 may be involved in augmenting LPS-induced signaling, potentially by incorporation of Hsp60 into the TLR4 signaling complex, by Hsp60 binding to other activating receptors, and/or by Hsp60 interacting with components of the LPS-signaling system.

Thus Cpn10 binding to Hsp60 may prevent Hsp60 interaction with cell surface receptors and thereby inhibit Hsp60-mediated augmentation of TLR4 signaling.

We also have evidence described herein that Hsp10 can also inhibit signaling by a TLR2 agonist. Perhaps the physiological role of circulating Hsp10 during early pregnancy (Morton,1998, *Immunol Cell Biol* 76, 483-496) may be to remove the Hsp60 danger signal that has arisen from pregnancy rather than from pathogen-induced tissue damage.

The ability to reduce, but not suppress completely, TNF-α secretion would distinguish Cpn10 from other anti-inflammatory therapies, particularly those based on anti- TNF-α antibodies, which can accomplish efficient removal of TNF-α. However, such removal may not always be desirable. For instance, TNF-α antibody treatment has been shown ultimately to increase the severity of multiple sclerosis (MS; Wiendl & Hohlfeld, 2002, BioDrugs **16**, 183-200). MS has been suggested as a possible therapeutic target for Cpn10 since Cpn10 is reported to reduce clinical signs and delay onset of disease in a murine model of MS (experimental autoimmune encephalomyelitis; Zhang et al., 2003, J Neurol Sci **212**, 37-46)

As described herein, the ability of Cpn10 to reduce the expression of inflammatory mediators indicates that Cpn10 may find therapeutic application in conditions where excessive LPS, TLR4,TLR3 and/or TLR2 signaling and/or Hsp60 leads to pathology.

Furthermore, this mode of action is not via tolerance induction or preferential activation/supression of Th1/Th2 responses.

It is also proposed that continuous infusion of Cpn10 may be a particularly advantageous mode of delivery that maximizes the therapeutic effects of Cpn10.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference.

DATED this sixteenth day of July 2004

CBIO LIMITED

by its Patent Attorneys

FISHER ADAMS KELLY

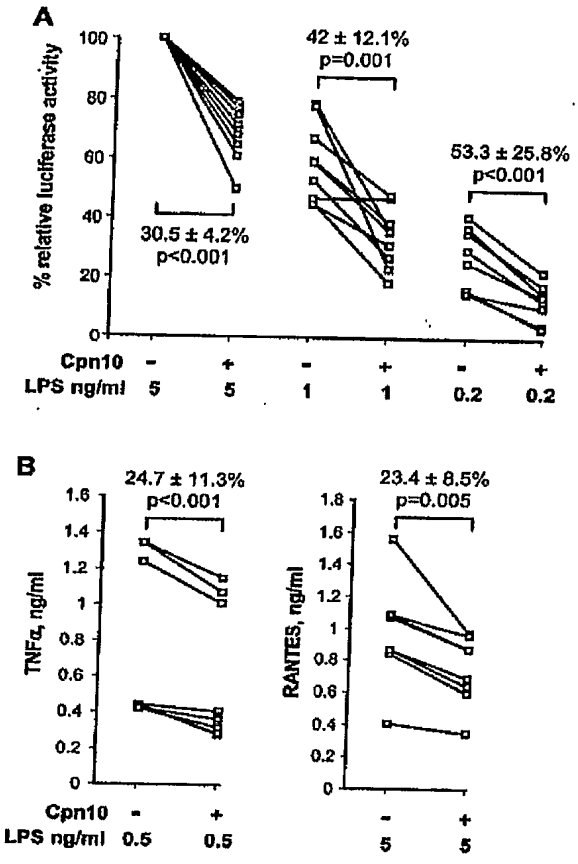

A
% relative luciferasa activity
100
80
60
40
20
0
30.5 ± 4.2%
p<0.001
42 ± 12.1%
p=0.001
53.3 ± 25.8%
p<0.001
Cpn10 - + - + - +
LPS ng/ml 5 5 1 1 0.2 0.2
B
TNFα, ng/ml
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
24.7 ± 11.3%
p<0.001
Cpn10 - +
LPS ng/ml 0.5 0.5
RANTES, ng/ml
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
23.4 ± 8.5%
p=0.005
- +
5 5

FIG. 1

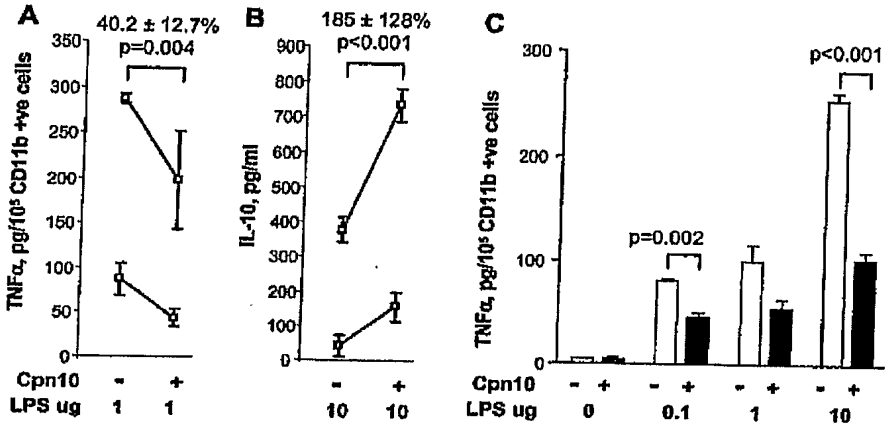

A
40.2 ± 12.7%
p=0.004
TNFα, pg/10⁵ CD11b +ve cells
350
300
250
200
150
100
50
0
Cpn10 - +
LPS ug 1 1
B
185 ± 128%
p<0.001
IL-10, pg/ml
900
800
700
600
500
400
300
200
100
0
- +
10 10
C
p<0.001
p=0.002
TNFα, pg/10⁵ CD11b +ve cells
300
200
100
0
Cpn10 - + - + - + - +
LPS ug 0 0.1 1 10

FIG. 2

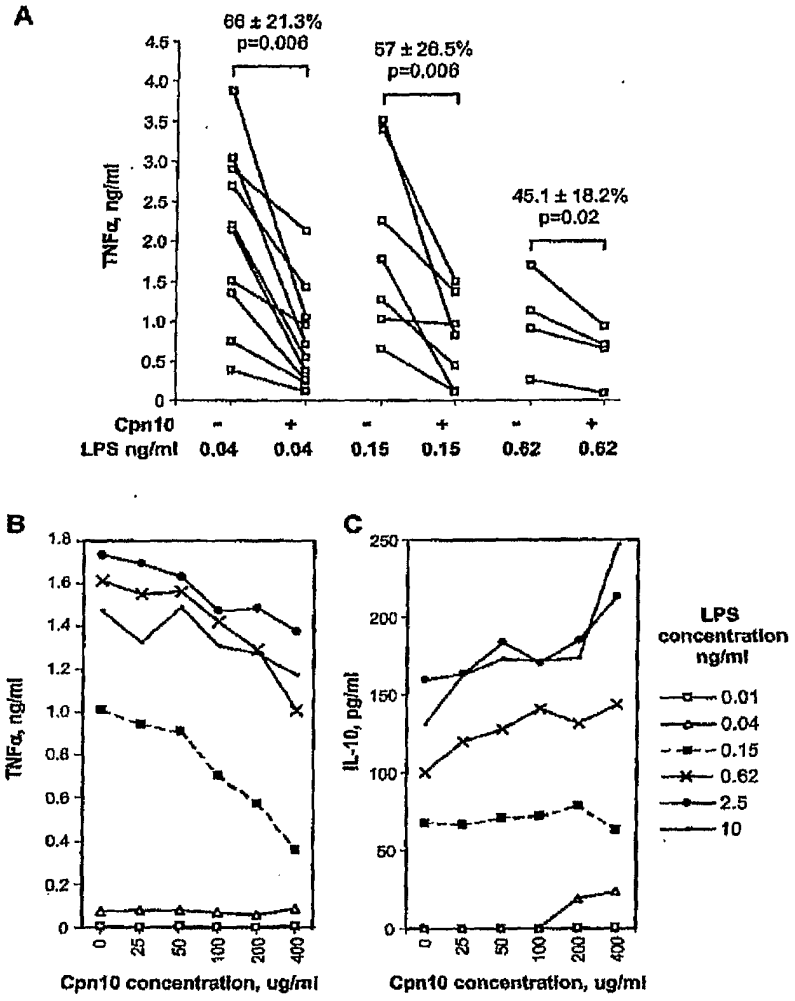

A
66 ± 21.3%
p=0.006
57 ± 26.5%
p=0.006
45.1 ± 18.2%
p=0.02
TNFα, ng/ml
Cpn10
LPS ng/ml
0.04
0.15
0.62
B
TNFα, ng/ml
Cpn10 concentration, ug/ml
C
IL-10, pg/ml
Cpn10 concentration, ug/ml
LPS concentration ng/ml
0.01
0.04
0.15
0.62
2.5
10

FIG. 3

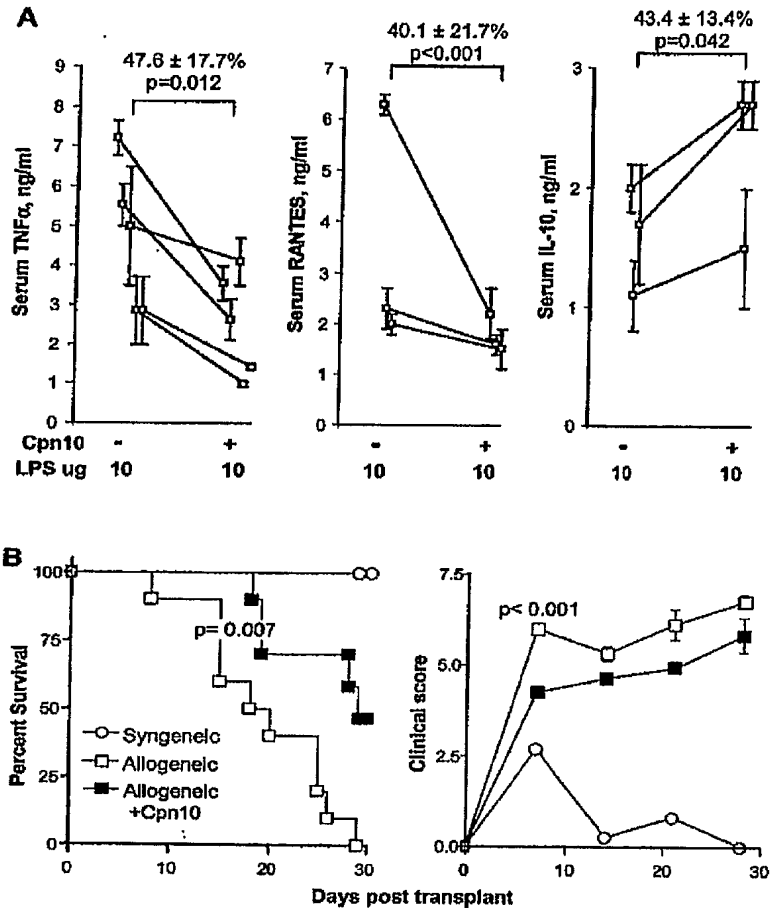

A
47.6 ± 17.7%
p=0.012
40.1 ± 21.7%
p<0.001
43.4 ± 13.4%
p=0.042
Serum TNFα, ng/ml
Serum RANTES, ng/ml
Serum IL-10, ng/ml
Cpn10 - +
LPS ug 10 10
B
Percent Survival
p= 0.007
Syngeneic
Allogeneic
Allogeneic +Cpn10
Clinical score
p< 0.001
Days post transplant

FIG. 4

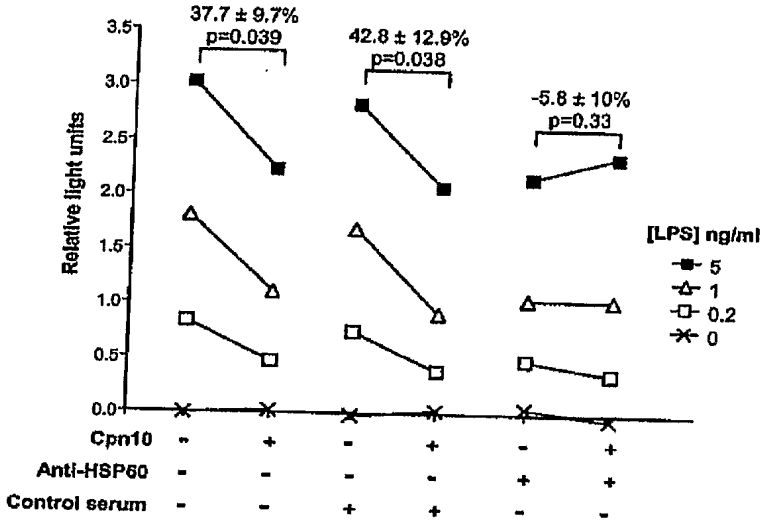

37.7 ± 9.7%
p=0.039
42.8 ± 12.9%
p=0.038
-5.8 ± 10%
p=0.33
Relative light units
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
[LPS] ng/ml
5
1
0.2
0
Cpn10 - + - + - +
Anti-HSP60 - - - - + +
Control serum - - + + - -

FIG. 5

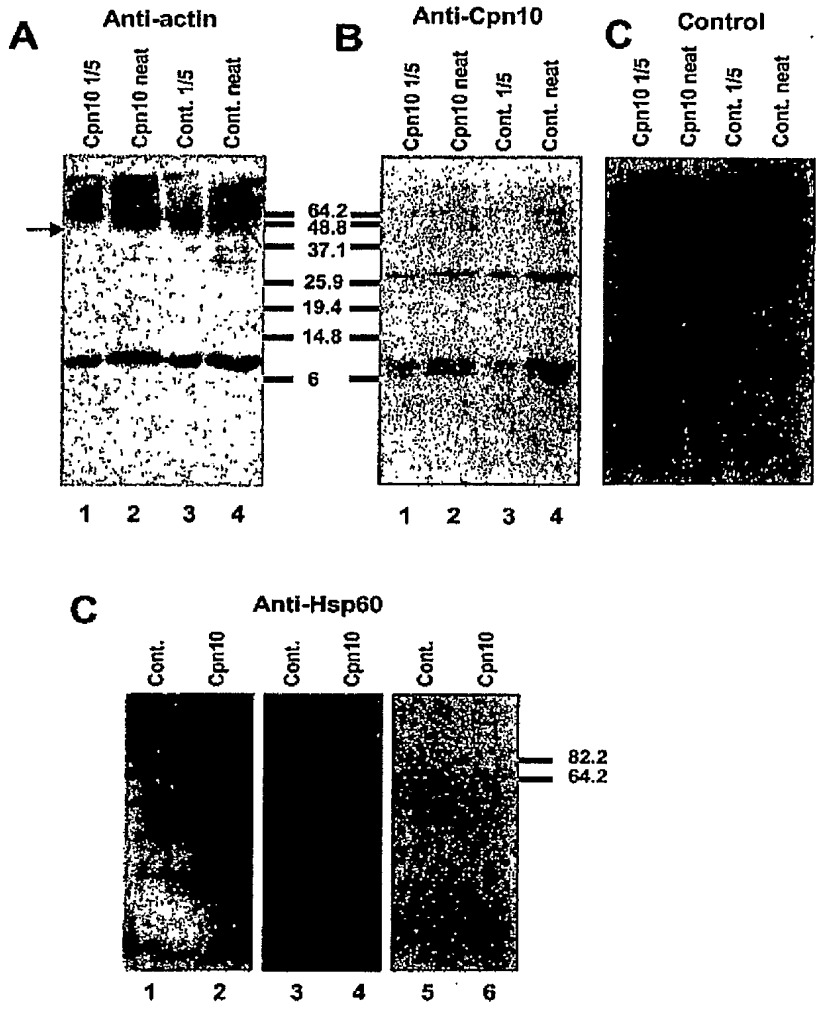

A
Anti-actin
Cpn10 1/5
Cpn10 neat
Cont. 1/5
Cont. neat
64.2
48.8
37.1
25.9
19.4
14.8
6
1 2 3 4
B
Anti-Cpn10
Cpn10 1/5
Cpn10 neat
Cont. 1/5
Cont. neat
1 2 3 4
C
Control
Cpn10 1/5
Cpn10 neat
Cont. 1/5
Cont. neat
C
Anti-Hsp60
Cont.
Cpn10
Cont.
Cpn10
Cont.
Cpn10
82.2
64.2
1 2 3 4 5 6

Figure 6

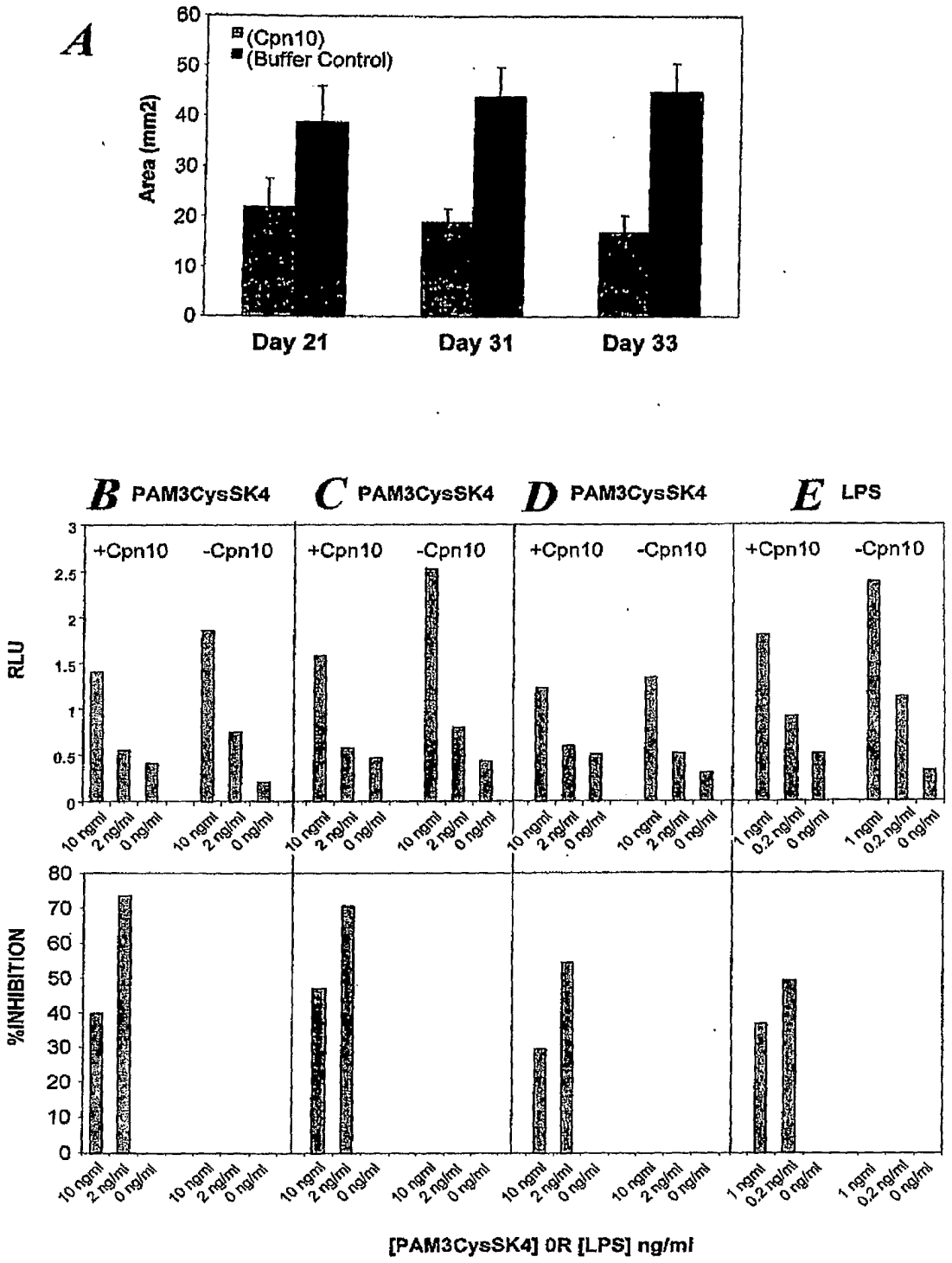

A
(Cpn10)
(Buffer Control)
Area (mm2)
60
50
40
30
20
10
0
Day 21
Day 31
Day 33
B PAM3CysSK4
C PAM3CysSK4
D PAM3CysSK4
E LPS
+Cpn10
-Cpn10
RLU
3
2.5
2
1.5
1
0.5
0
10 ngml
2 ng/ml
0 ng/ml
1 ngml
0.2 ng/ml
%INHIBITION
80
70
60
50
40
30
20
10
0
[PAM3CysSK4] 0R [LPS] ng/ml

FIG. 7

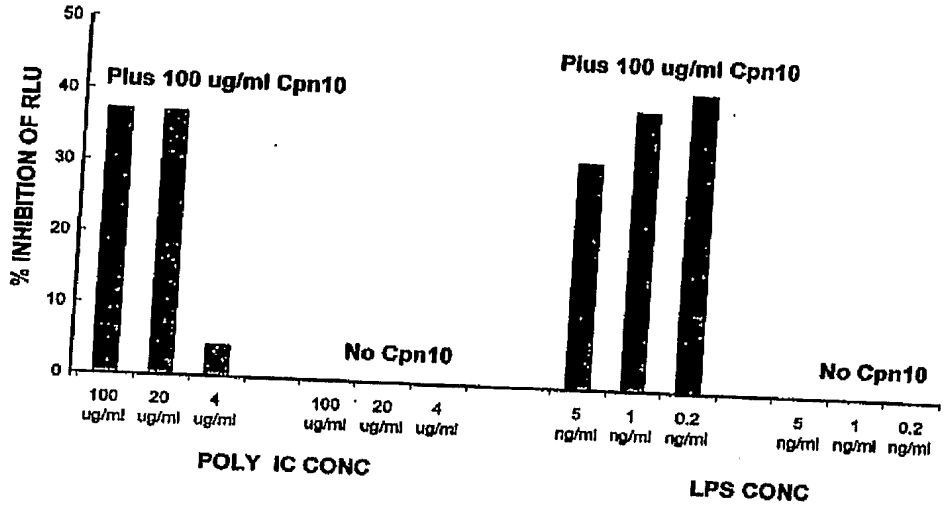

% INHIBITION OF RLU
50
40
30
20
10
0
Plus 100 ug/ml Cpn10
No Cpn10
Plus 100 ug/ml Cpn10
No Cpn10
100 ug/ml
20 ug/ml
4 ug/ml
100 ug/ml
20 ug/ml
4 ug/ml
5 ng/ml
1 ng/ml
0.2 ng/ml
5 ng/ml
1 ng/ml
0.2 ng/ml
POLY IC CONC
LPS CONC

FIG. 8

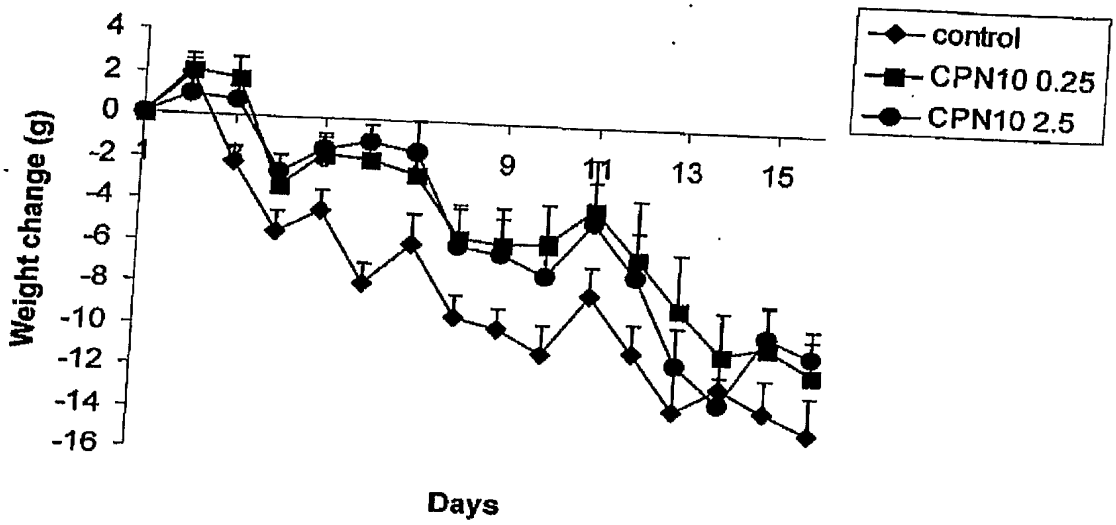

control
CPN10 0.25
CPN10 2.5
Weight change (g)
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
9
11
13
15
Days

FIG. 9

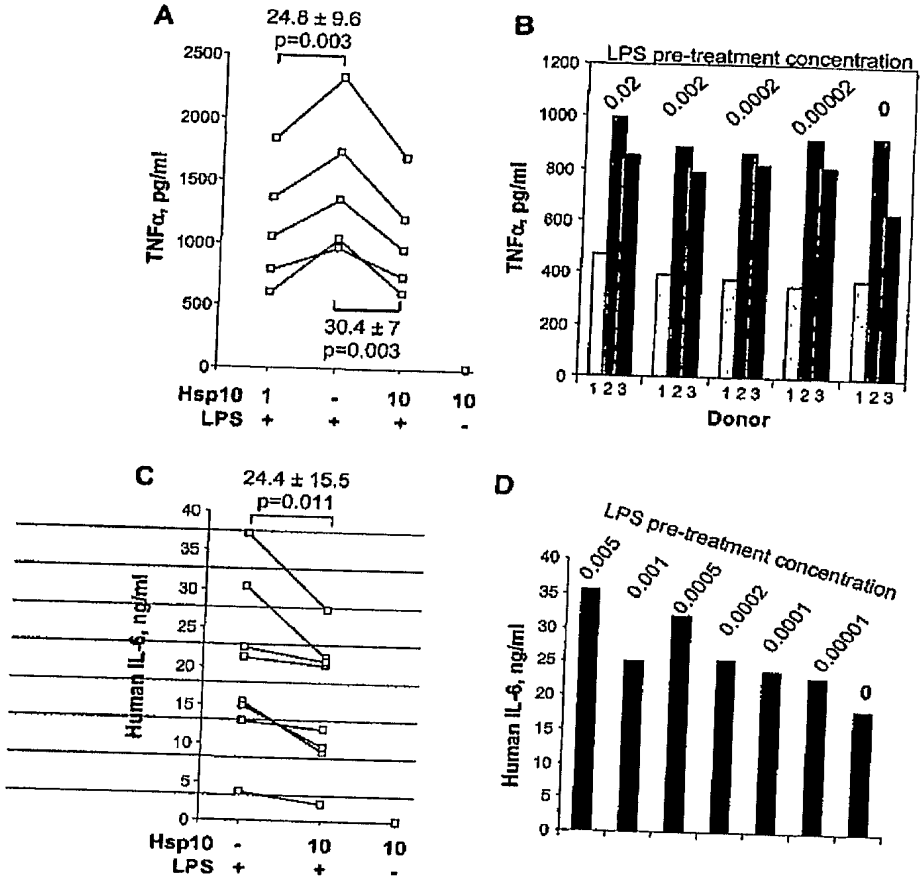

A
24.8 ± 9.6
p=0.003
TNFα, pg/ml
2500
2000
1500
1000
500
0
30.4 ± 7
p=0.003
Hsp10 1 - 10 10
LPS + + + -
B
LPS pre-treatment concentration
TNFα, pg/ml
1200
1000
800
600
400
200
0
0.02
0.002
0.0002
0.00002
0
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
Donor
C
24.4 ± 15.5
p=0.011
Human IL-6, ng/ml
40
35
30
25
20
15
10
5
0
Hsp10 - 10 10
LPS + + -
D
LPS pre-treatment concentration
Human IL-6, ng/ml
40
35
30
25
20
15
10
5
0
0.005
0.001
0.0005
0.0002
0.0001
0.00001
0

FIG. 10

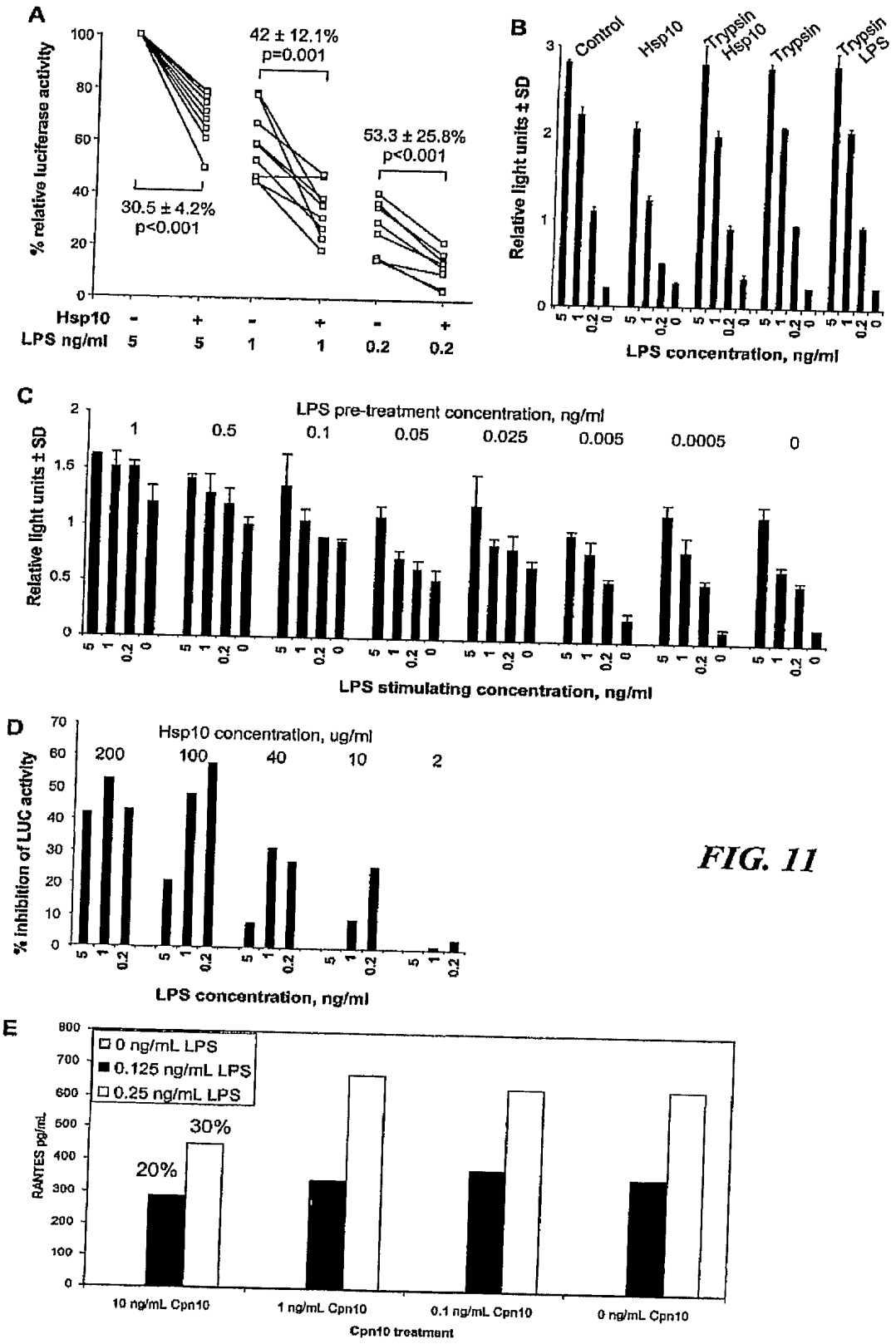

A
% relative luciferase activity
42 ± 12.1%
p=0.001
53.3 ± 25.8%
p<0.001
30.5 ± 4.2%
p<0.001
Hsp10
LPS ng/ml
B
Control
Hsp10
Trypsin Hsp10
Trypsin
Trypsin LPS
Relative light units ± SD
LPS concentration, ng/ml
C
LPS pre-treatment concentration, ng/ml
Relative light units ± SD
LPS stimulating concentration, ng/ml
D
Hsp10 concentration, ug/ml
% inhibition of LUC activity
LPS concentration, ng/ml
E
0 ng/mL LPS
0.125 ng/mL LPS
0.25 ng/mL LPS
RANTES pg/mL
20%
30%
10 ng/mL Cpn10
1 ng/mL Cpn10
0.1 ng/mL Cpn10
0 ng/mL Cpn10
Cpn10 treatment

FIG. 11

FIG. 12A

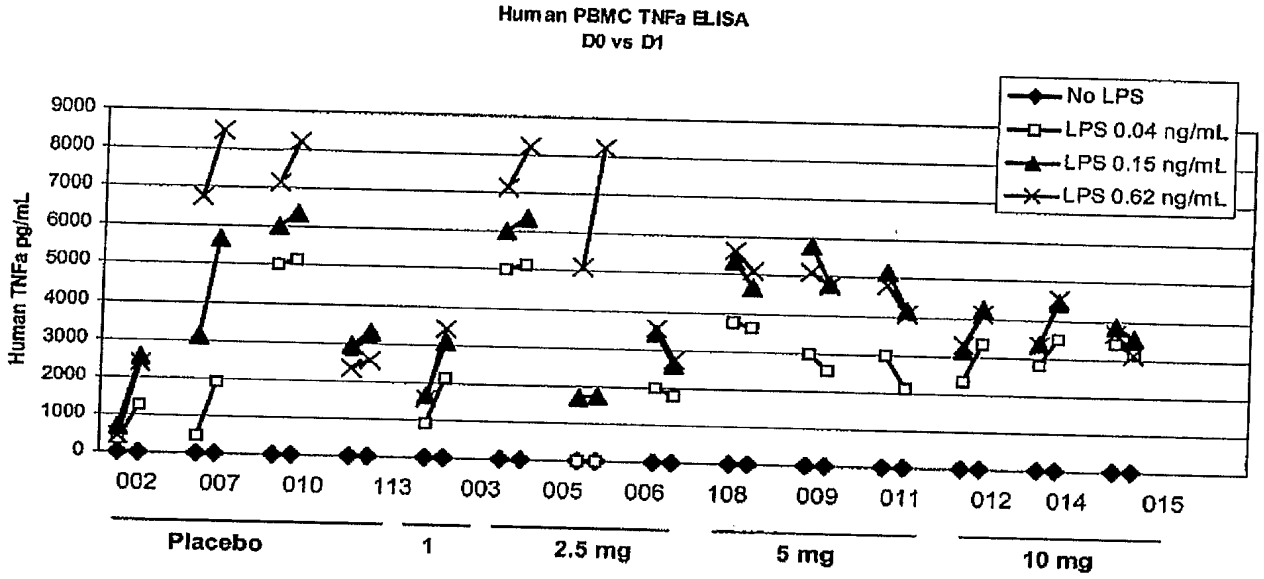

Human PBMC TNFa ELISA
D0 vs D1
Human TNFa pg/mL
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
No LPS
LPS 0.04 ng/mL
LPS 0.15 ng/mL
LPS 0.62 ng/mL
002
007
010
113
003
005
006
108
009
011
012
014
015
Placebo
1
2.5 mg
5 mg
10 mg

FIG. 12B

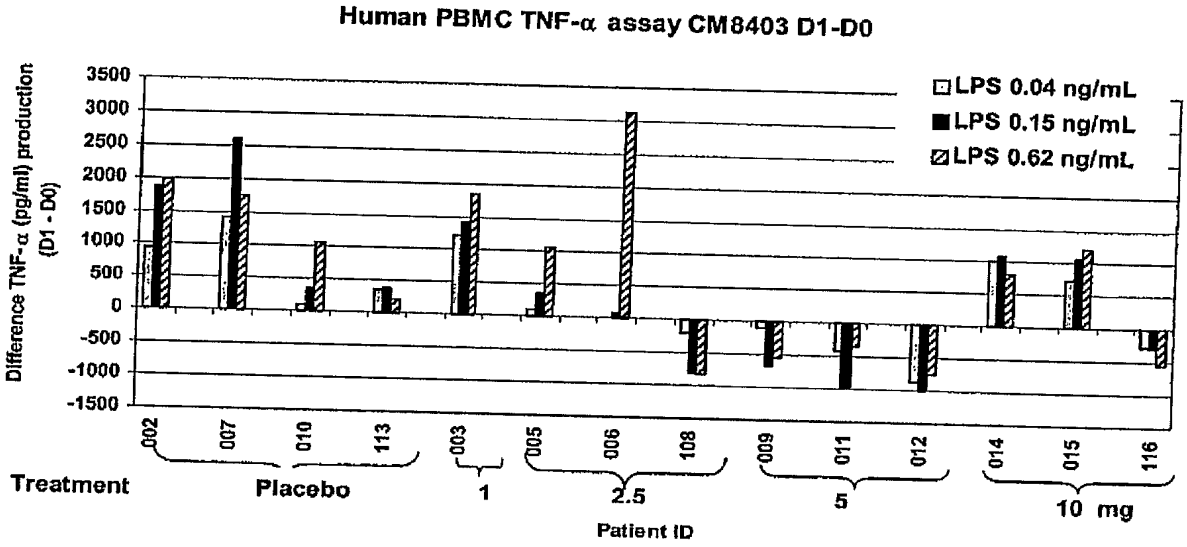

Human PBMC TNF-α assay CM8403 D1-D0
Difference TNF-α (pg/ml) production (D1 - D0)
3500
3000
2500
2000
1500
1000
500
0
-500
-1000
-1500
LPS 0.04 ng/mL
LPS 0.15 ng/mL
LPS 0.62 ng/mL
002
007
010
113
003
005
006
108
009
011
012
014
015
116
Treatment
Placebo
1
2.5
5
10 mg
Patient ID

FIG. 12C

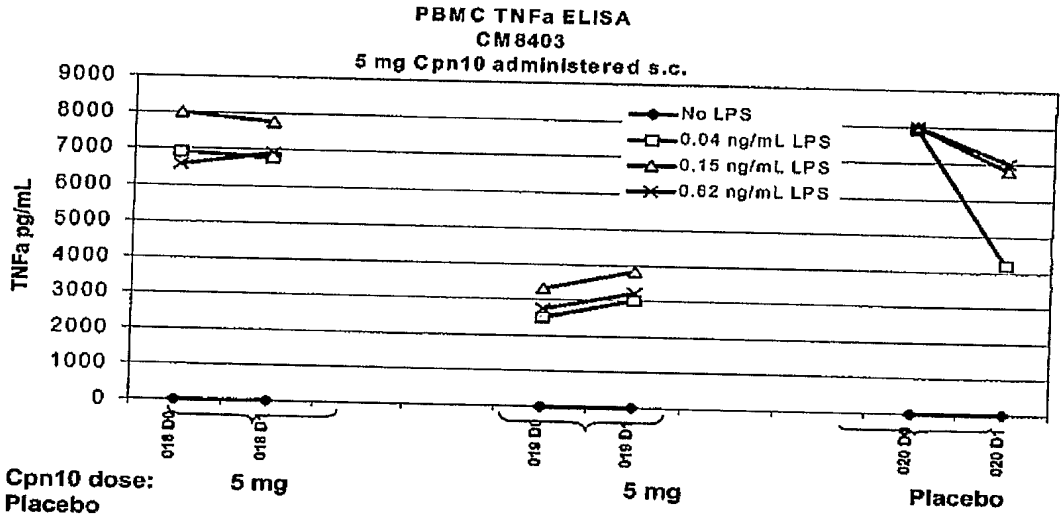

PBMC TNFa ELISA
CM8403
5 mg Cpn10 administered s.c.
TNFa pg/mL
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
No LPS
0.04 ng/mL LPS
0.15 ng/mL LPS
0.62 ng/mL LPS
018 D0
018 D1
019 D0
019 D1
020 D0
020 D1
Cpn10 dose:
Placebo
5 mg
5 mg
Placebo

Figure 13

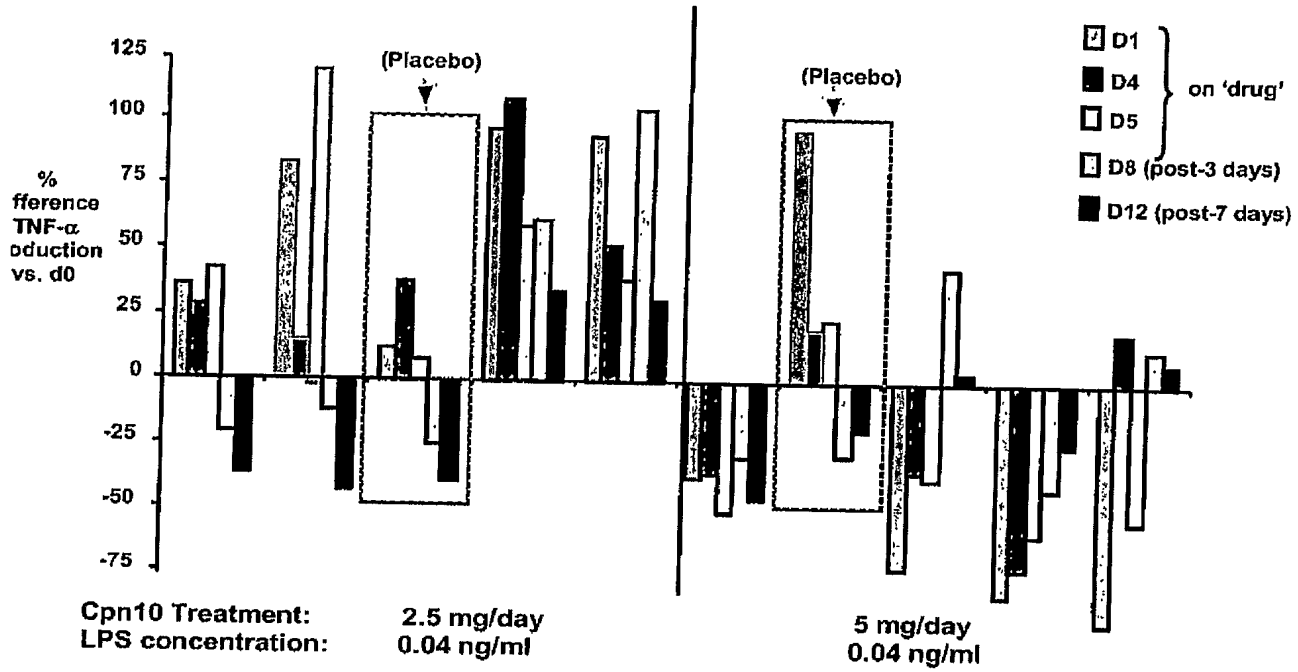
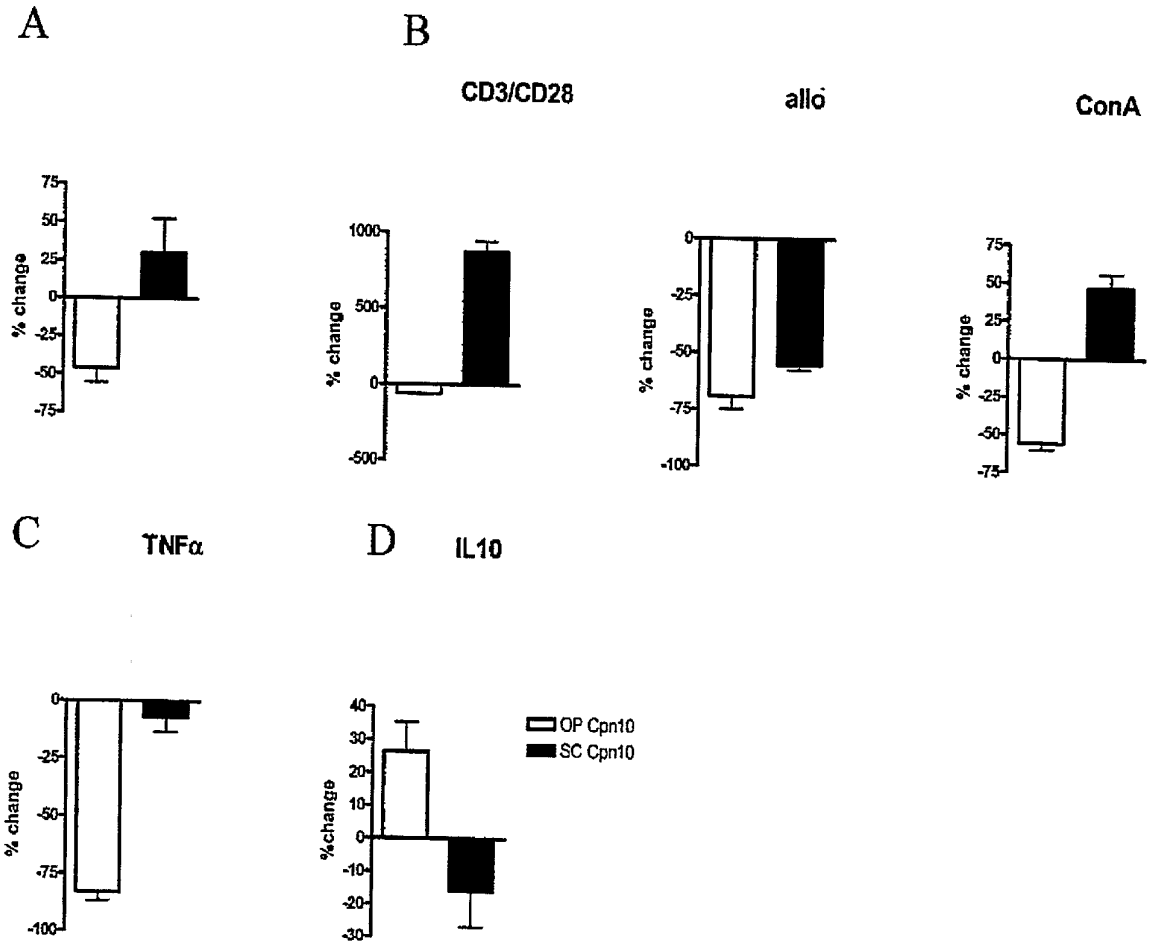

125
100
75
50
25
0
-25
-50
-75
% fference TNF-α oduction vs. d0
(Placebo)
(Placebo)
D1
D4
D5
on 'drug'
D8 (post-3 days)
D12 (post-7 days)
Cpn10 Treatment:
LPS concentration:
2.5 mg/day
0.04 ng/ml
5 mg/day
0.04 ng/ml

Figure 14


A
B
CD3/CD28
allo
ConA
C
TNFα
D
IL10
% change
%change
OP Cpn10
SC Cpn10